PB82-230889

Virion Aggregation and Disinfection of Water by Chlorine and Bromine

North Carolina Univ. at Chapel Hill

Prepared for

Municipal Environmental Research Lab.
Cincinnati, OH

Mar 82

U.S. Department of Commerce
National Technical Information Service
NTIS

PB82-230889

600/2-82-022

VIRION AGGREGATION AND DISINFECTION OF WATER BY CHLORINE AND BROMINE

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Grant No. R-804587

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NATIONAL TECHNICAL
INFORMATION SERVICE
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SPRINGFIELD, VA 22161

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO.
EPA-600/2-82-022

2.
ORD Report

3. RECIPIENT'S ACCESSION NO.
PB82-236889

4. TITLE AND SUBTITLE
Virion Aggregation and Disinfection of Water by Chlorine and Bromine

5. REPORT DATE
March 1982

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
D. G. Sharp

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Department of Bacteriology and Immunology
University of North Carolina
Chapel Hill, North Carolina 27514

10. PROGRAM ELEMENT NO.
BNC1A

11. CONTRACT/GRANT NO.
R-804587

12. SPONSORING AGENCY NAME AND ADDRESS
Municipal Environmental Research Laboratory - Cinti, OH
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

13. TYPE OF REPORT AND PERIOD COVERED
Final

14. SPONSORING AGENCY CODE
EPA/600/14

15. SUPPLEMENTARY NOTES
Project Officer: John C. Hoff (513) 684-7331

16. ABSTRACT

The state of aggregation among virions suspended in water was measured and quantitatively characterized by several methods developed in this research. All unpurified virus suspensions contained some aggregates and all of them contained half or more singles. Stable purified suspensions of single virions without detectable aggregation were prepared and used for comparison of inactivation kinetics of several viruses under a variety of conditions. All of the viruses tested tended to aggregate at acid pH, but the pH below which aggregation began was quite different for different viruses. All the viruses tended to aggregate at low ionic strength, but the kind of salt or buffer present strongly influenced the rate of aggregation at a given pH and temperature. Virion aggregation caused departures from first order inactivation kinetics. However, monodisperse suspensions of certain viruses also showed similar departures apparently related to inactivation mechanisms. Reversal of inactivation of monodisperse inactivated echovirus by complementation also was demonstrated. Inactivation of poliovirus by OCl^- at pH 10 was very weak compared with HOCl at pH 6. Increasing sensitivity of the virus to HOCl as pH was increased to 7, 8, and 9 appears to be due to a change in the virus rather than to OCl^-. Addition of 0.1M NaCl at pH 10 increased the inactivation rate more than 100-fold. Chlorides of cesium and potassium enhanced the rate, also making it comparable with that of HOCl at pH 6.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT
Release to Public

19. SECURITY CLASS *(This Report)*
Unclassified

20. SECURITY CLASS *(This page)*
Unclassified

21. NO. OF PAGES
122

22. PRICE

EPA Form 2220-1 (9-73)

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research, a most vital communication link between the researcher and the user community.

The research described in this report contributes substantially to our knowledge of the effects of enterovirus aggregation on the resistance of these viruses to inactivation by disinfection. The effects of physical and chemical factors on aggregation also were studied. The information obtained is of interest to all investigators concerned with microbiological aspects of drinking water treatment.

Francis T. Mayo, Director
Municipal Environmental Research
Laboratory

ABSTRACT

This research work has provided examples of the effect of virion aggregation on the inactivation of several viruses by chlorine and bromine in water. In all cases, aggregation has increased the survival time; and when the survival ratio was very low, the increase was very great. This is no longer qualitative speculation; methods are described here by which the state of aggregation (the colloidal state) of a virus particle suspension can be measured and numerically characterized. Methods are described also for accurately measuring virus inactivation rates even in those cases where contact time was as short as 0.5 second. In this way, initial reaction rates have been observed, and departures from first order kinetics are clearly shown in some cases to be caused by virion aggregation. Many other examples have been just as clearly shown to be quite unrelated to virion aggregation.

Meaningful comparisons of the sensitivity of different viruses to chlorine or bromine or of a given virus and halogen agent under different conditions of temperature, pH, etc. were made here, as they must be, under identical states of aggregation. Inasmuch as the only reproducible state is zero aggregation, all effort was made to achieve it, and clear evidence from the electron microscope and/or the ultracentrifuge has been presented to show to what extent it was achieved.

At pH 7, poliovirus (Mahoney) was only slightly more sensitive to bromine than it was to chlorine at pH 6; but at pH 10, bromine was more than 40 time more effective than chlorine. Poliovirus (Brunhilde) was twice as resistant to 20 μM HOCl as the Mahoney virus, and the two coxsackie viruses B3 and B5 were about twice that of Brunhilde.

All the viruses tested tended to aggregate at acid pH, but the pH below which aggregation began was quite different for different viruses. All the viruses tended to aggregate at low ionic strength, but the kind of salt or buffer present strongly influenced the rate of aggregation at a given pH and temperature. The rates of aggregation at low pH were found to be in good agreement with the rapid coagulation theory devised for colloids 63 years ago.

Echovirus (Farouk) departed radically from linear semi log kinetics of inactivation by HOCl. After an instantaneous 2-log drop in plaque titer, there was no further action for 15 seconds, after which a slow, steady decline set in. Some similar effects were seen also with the coxsackie virus. Echovirus plaque titer could be partially revived (after treatment with HOCl) by inducing aggregation among the virions. Suvival titer could

sometimes be revived over 1000-fold by this process. Such revival after chlorine treatment has never been reported before.

The inactivation of poliovirus by OCl^- at pH 10 was very weak compared with $HOCl$ at pH 6. Increasing sensitivity of the virus to $HOCl$ as pH was increased to 7,8, and 9 appears to be due to a change in the virus rather than to OCl^-. However, addition of 0.1M $NaCl$ at pH 10 increased the inactivation rate more than 100-fold. Chlorides of cesium and potassium enhanced the rate, also making it comparable with that of $HOCl$ at pH 6.

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ABBREVIATIONS

PFU --- Plaque forming unit.

IR --- Inactivation rate (Log$_{10}$ PFU/time).

PTA --- Phosphotungstic acid.

EM --- Electron microscope.

VAT --- Virion aggregation test.

SPA --- Single particle analysis.

mM --- Millimolar.

μM --- Micromolar.

KA --- Kinetic attachment (preparation method for suspended particles for quantitative EM evaluation).

SD --- Spin down (preparation method for suspended particles for quantitative EM).

MR --- Multiplicity reactivation.

MA --- Multiplicity activation.

VP --- Virus particle or virion.

pI --- pH at which electrophoretic mobility is zero.

RNA --- Ribonucleic acid.

ACKNOWLEDGEMENTS

This work was supported mainly by research grant R803771 from the U.S. Environmental Protection Agency and in part by the U.S. Army Medical Research and Development Command under contract DAMD17-17-A013. It has been materially aided by Mr. Richard Boynton of the Sperry-Rand Company of Durham, N.C., who provided the special motor windings for the magnetic mixer devices used in the continuous-flow disinfection apparatus.

The oil-driven OTD2 ultracentrifuge with the ARC-1 slow start device were gifts of the Ivan Sorvall Company of Newtown, Conn.

Most of the experimental work was done by Roger Floyd, Dorothy Young, Harold Jensen, Kent Thomas and Jenny Leong. Quantitative electron microscopy (virion counting and aggregation analysis) was done by the author with the excellent technical assistance of Florence Stubbs and later by Jenny Leong.

All of the devices for preparations of virus for quantitative electron microscopy (KA apparatus, etc.) as well as the continuous-flow apparatus were made by the author in his home machine shop.

Dr. J.D. Johnson, whose name is on several of the published papers, has provided constant guidance in all situations involving halogen chemistry.

Dr. John C. Hoff, Project Officer, deserves special mention here. His wide knowledge of public health virology and his quick recognition of the value of newly devised methods for solving new problems together with the constant encouragement of the investigator who must solve them combine to place him very high in this author's merit list of public servants.

INTRODUCTION

When the discovery is made that the result of a chemical reaction is a desirable compound or, in this case, the inactivation of an infectious virus, the next step is to investigate the kinetics of the reaction. the immediate practical value of such time studies is the accumulation of useful data on how much inactivation can be achieved in a given time, and the long range basic gain may be an understanding of the mechanism of the reaction which may be a greater ultimate value than early factual data.

Inasmuch as disinfection (as usually observed) involves the reaction of relatively few virus particles mixed with many molecules of the active agent, it is reasonable to expect that the number of infective units destroyed per unit time might be proportional to the number that have survived up to that time. These are the conditions that the physical chemist will recognize as those that indicate a monomolecular reaction (that is, one characterized by a linear decline in the logarithm of the infectivity plotted against time). When virus infectivity is measured in terms of plaque-forming units (PFU), the slope of the line formed by plotting log PFU versus time is the inactivation rate (IR).

Although it is true that a monomolecular reaction produces a straight line and virus inactivation sometimes produces a straight line, there are many data from carefully conducted virus experiments that have produced inactivation curves. Several investigators in search of the reason for this have suggested that aggregation among the virions could produce departure from linearity and add substantially to the survival time of infectivity in a water suspension of virus to which a halogen disinfecting agent has been added. The reasoning has usually been that clumping of the virions must reduce the accessibility of some of them to the disinfecting agent and that this effect must become greater with increasing clump size. Unless all the clumps are the same size, one would expect the infectivity of a water suspension of single virions mixed with clumps of many sizes to decrease rapidly at first contact with a disinfecting agent, but when all single particles have been destroyed and the surviving PFU are successively larger clumps, the IR must decrease, producing the inactivation curve. It must not be assumed that all inactivation curves would be straight lines were it not for virion aggregation; but the idea seemed worth considering, if only briefly, at the beginning of this work.

The lack of data or of any attempt to express in quantitative terms the colloidal state of a virus particle suspension has limited to speculation any correlation between aggregation and IR, and it is this question that this research project was designed to clarify.

Two techniques (1) have been successfully employed in this work with the electron microscope (EM), by which the number of single virions and the number of aggregates of each size in a virus suspension can be counted. Thus a histogram can be constructed completely characterizing the state of aggregation of the virus particles. The EM techniques have been very useful when only a few aggregates are present, and they are indispensible as a quick check for trace of aggregation in stock virus preparations for IR experiments. But when aggregation involves much over 20% of the virions, complete characterization by EM becomes impractical, and the centrifuge has been employed to better advantage. Two methods of this kind have been devised and used here. The virus aggregation test (VAT) gives a quick and easy qualitative separation of the PFU in a mixed suspension into single, small, and large aggregates. The single particle analysis (SPA) is much more precise if it is the fraction of single particles that must be determined, and it can be determined even in very dilute suspensions as long as there is enough virus present to be detected by the plaque method. The SPA technique is particularly useful for determining the rate of aggregation. The data provide a *numerical* measure of the physical stability or instability of a virus suspension as a function, for example, of pH or salt concentration.

After characterizing the colloidal state of a virus preparation on a quantitative basis for correlation with IR data, our next aim was to compare the halogen resistance of several picornaviruses. This has proved to be difficult from a practical point of view, and some of the difficulties have been revealed in our first report (1) and in otherwise published papers covering early experiments (2,3,4,5). Electron microscope examination of our first virus preparations for the IR work indicated that aggregation was always present unless more than usual care was taken in preparation and storage of the stock virus. In fact, a major part of the first year's work (as covered in our first report) was spent in establishing the methods for preparing and storing essentially aggregation-free, purified virus with low enough halogen demand for IR experiments. Before this was finally accomplished we came to suspect that much of the IR work done with virus that had not been so carefully prepared to avoid aggregation must have yielded rates characteristic of the state of aggregation rather than of the virions under study.

In spite of many attempts, we were never able to reproduce virus suspensions with a given degree of aggregation, nor were we able to prevent the aggregation of a high-titer preparation from slowly changing. One suspects that there must be ways of doing this, but we did not find them. result was that no series of IR experiments could be done with virus in any particular state of aggregation. The only reproducible state of suspension was monodisperse (all singles), so all the comparative IR experiments were done with virus shown by the EM to be in this condition--with one exception. Coxsackie B5 virus could not be kept monodisperse after purification.

The aim of this research is a study of virion aggregation and its effect on the inactivation of virus by halogen treatment of water. Though the title does not specify salt water, it is difficult to conceive of laboratory experiments without some means of maintaining pH. At the outset, it was apparent that the active form of the free bromine or chlorine would be determined primarily by pH, and to a lesser extent by any salts that were present. Later on, these two variables were found to govern virion aggregation as well. Still later, their vital involvement with IR (particularly at high pH) was discovered. A few experiments have been done with unbufferd water, but most of them were done with carefully buffered suspending solutions so that other investigators could repeat them accurately if they wished. For a complete account of the experiments and results of this project, the earlier report (1) or references 2 through 5 must be read first. They describe in detail the continuous flow apparatus that was designed and built for this work and in which plaque survival ratios can be determined for virus whose contact time with the free halogen agents in water may be short as 0.5 second. Preparation, purification, plaquing, physical assay (bv EM), and storage of virus suspensions for IR experiments are described, and they have not been changed except as noted in the materials and methods section of this final report. In addition to early experiments demonstrating the effects of virion aggregation on the IR of reovirus by bromine (HOBr) and poliovirus by chlorine (HOCl), the first report deals with the natural aggregates found in some dialyzed but not otherwise purified preparations of poliovirus as well as some observations of aggregate formation when purified stock virus preparations were diluted with water. This report begins with the extensive gathering of IR data on poliovirus, and it continues with experimental data on rates of aggregation and practical as well as theoretical measures of virion colloidal stability. Finally, after the salt effects on IR were discovered, the remaining time and effort was largely devoted to learning how general this remarkable effect might be.

CONCLUSIONS

1. The survival of infectivity in some halogen-treated aggregated preparations of virus is several orders of magnitude greater than that in similarly treated monodisperse preparations of the same virus.

2. Aggregated virus diluted with water and virus released from infected cells directly into water by freeze-thaw cycles did not become dispersed as single virions; most of the aggregates remained.

3. Poliovirus (Mahoney and Brunhilde), echovirus, reovirus and coxsackie B3 all remained well dispersed in dilute buffers in the pH range 7 to 10 even after purification and concentration to 10^{11} virions/ml. Only coxsackie B5 quickly became aggregated under these conditions. All of them aggregated rapidly in the acid range but the highest pH at which aggregation began was different for different viruses.

4. Whenever purified virus concentrates were diluted in water, aggregation started. The rate increased rapidly with increase in virus concentration.

5. Theoretical coagulation rates predicted 63 years ago for colloids in general are shown here to apply to purified reovirus and poliovirus as well.

6. Addition of salts to water generally improved suspension stability of the purified viruses. In the acid range sodium chloride was very useful. Low concentrations of $CaCl_2$ and $MgCl_2$ were more effective than NaCl but at higher concentrations these salts caused aggregation of some viruses.

7. Different buffers at the same pH were very different in their ability to maintain purified virions in suspension.

8. Removal of aggregation from a virus particle suspension did not in general result in a constant inactivation rate in the presence of either chlorine or bromine. The graph of log PFU vs. time was generally not straight even with monodisperse preparations. A most conspicuous example of this was seen in the complex reaction kinetics of echovirus with chlorine.

9. The inactivation rate of poliovirus (Mahoney) increased with pH from 6 to 9 at constant HOCl concentration but at pH 10 where most of the free chlorine was OCl^- the rate was very slow. Apparently, the

virus sensitivity to HOCl increases as pH approches 10.

10. Addition of 0.1 M NaCl to dilute buffers increased the disinfecting effect of HOCl for two poliovirus at pH 6 by a factor of 2 to 3X and at pH 10 the OCl^- rate was 30 to 150 fold greater when this salt was added.

11. Addition of 0.1 M NaCl had no effect on the reaction rate of HOCl at pH 6 on either the monodisperse coxsackie B3 or on the always aggregated coxsackie B5 but it greatly increased the rate for both with OCl^- at pH 10.

12. The inactivation rate of OCl^- on coxsackievirus B5 was greatly increased by all three salts NaCl, CsCl and KCl but not equally. The effect of the cesium salt was least, sodium was greater and KCl was the most effective.

13. The complex inactivation kinetics observed with HOCl and the echovirus was not unique. Lesser effects of the same kind were observed with the coxsackie B5 further suggesting that a metastable state of capsid protein conformation must be considered in forming a more complete concept of the reaction of chlorine with these possibly other picornaviruses.

14. The remarkable 3000 fold reactivation of echovirus infectivity after reduction by a factor of 10^5 by HOCl showed that subsequent aggregation by mild acid treatment could greatly increase the plaque survival ratio. There is nothing in this work that shows that there is such a revival of infectivity for animals or man but the suggestion is inescapable.

15. Among the 5 closely related picornaviruses tested for their reaction with chlorine in water there were great differences in their aggregating behavior, in their rates of inactivation and in their response in the presence of salts particularly at high pH.

16. A straight line inactivation chart does not indicate that there is no virion aggregation. Some straight lines have been seen when aggregation was clearly demonstrated.

17. It has become clear from this work on viral disinfection of water that many more variables are present than were previously suspected. Therefore, we strongly urge that in the future all workers in viral disinfection control for and clearly state in the published paper the following variables: (i) the time of contact of the virus with the disinfectant, (ii) the temperature of the reaction, (iii) the total ionic strength of the reaction mixture, (iv) the pH of the reaction mixture, (v) the type of buffer used to maintain the pH, (vi) the total chlorine concentration and the relative amounts of HOCl and OCl^-, and (vii) the state of aggregation of the virus. Only when all of these parameters are known will it be possible to make adequate comparisons between data from different laboratories.

RECOMMENDATIONS

1. All of the virus inactivation experiments here were made after the removal of foreign matter that might compete with the virions for the active free halogen agent. This was necessary here but other, more difficult to control, experiments must be made in the presence of the halogen demanding materials normally found in polluted waters.

2. The effect of salts, other than the 3 examined here, on the chlorine reaction must be tested further particularly those found in hard waters and particularly at high pH.

3. The complex kinetic inactivation phenomenon observed with the echovirus would seem to be a means of learning more about the structure of viruses and their reaction with halogens. A further search should be made to see how general this behavior is.

4. The thousand fold reactivation that was observed here when a chlorine damaged suspension of single virions was induced to aggregate, was probably not a repair mechanism but rather a synergistic effect by which variously damaged virions complement each other making a clump more infectious than all of particles acting separately. This phenomenon must be examined further. It is the first evidence of apparent reversal of the viricidal effect of chlorine and it is a potentially dangerous thing.

5. Until much more is learned about the mechanism of inactivation of viruses by halogen agents in water it is often impractical to compare two viruses in terms of their rates of inactivation because one or both of the rates may vary with time of contact. A more practical comparison is possible in terms of the times necessary to reach a given survival level; preferably several different survival levels should be examined. This is not the method most useful to the scientist but it gives the engineer definite answers.

6. A practical decision must be made for each water purification project regarding the virus survival ratio that can be tolerated (zero risk is probably unreachable). The influence of virion aggregation is apparently different for different viruses but one can generalize to this extent. As the requirement is made more stringent from 90 to 99 to 99.9% etc. inactivation the influence of aggregation will increase at each step by a factor that increases with each step. If this general rule is kept in mind, the end result will doubtless contain fewer catastrophic surprises.

7. Among the people who have participated in the work described here, there are those with excellence in Virology, Physics, Halogen Chemistry and Mechanical Engineering. This mixture of experience is highly desirable and I believe, essential to the success of future investigations in this field.

MATERIALS AND METHODS

Preparation of purified virus for aggregation and IR experiments, preparation of glassware and measurement of free chlorine and bromine concentrations have been continued by the methods as described in the previous report (1). Physical assay (particle and aggregate count) and by EM and plaque assay remain unchanged. The continuous flow apparatus has been slightly altered. The weight-driven injection syringe (Fig. 1 of that report) has been replaced by a synchronous motor drive that gives better assurance of a constant injection rate.

A wide range phosphate-carbonate buffer was used in several experiments. Its composition and ionic strength values are given in Table 1 for the pH range 6 to 10.

TABLE 1. Composition of phosphate-carbonate buffer

pH	KH_2PO_4 (M)	Na_2CO_3	Ionic strength
6.0	0.0176	0.0012	0.021
7.0	0.0116	0.0042	0.024
8.0	0.0079	0.0061	0.026
9.0	0.0067	0.0067	0.027
10.0	0.0045	0.0078	0.026

Preparation of chlorine treated virus for EM observation of structural damage has been more difficult than expected but the following technique has proved successful. Virus particles were attached in the desired numbers to aluminum coated collodion films in the kinetic attachment (KA) apparatus (6) then chlorine solution was applied for the desired time interval followed by sodium thiosulphate solution to stop the reaction. When such preparations were dried and phosphotungstic acid (PTA) applied for negative staining, most of the virus was dislodged and lost but if filter paper was applied briefly to the edge of the wet grid only a very thin film of water remained. If 2% PTA was sprayed immediately onto this still wet surface with a throat sprayer (Devilbiss #42) the droplets were spread by the residual water film but there was not enough liquid present to wash the virus off the film. Strict adherence to this procedure has produced excellent pictures of negatively stained virus in various stages of degradation. Of course any aggregation seen in these pictures is not likely to be that of the liquid suspension from which they were deposited.

Isoelectrophoresis was performed in an LKB 8101 Ampholine column of 110 ml with 1% ampholines. The sucrose gradient used as a stabilizer was 5 to 40%. The virus sample, 0.5 X 10^5 to 1.5 X 10^5 plaque forming units, was added to the 5% sucrose-ampholine solution in the gradient maker before forming the gradient; this placed the virus throughout the entire ampholine column before establishing the electric field. The starting voltage and current were approximately 620 V and 11 mA, respectively, for a pH gradient of 3.5 to 10 and 670 V and 5.5 mA for a pH gradient of 7 to 9. As the current dropped over a period of 2.5 to 4 h, adjustments in the voltage were made at 15-to 30-minute intervals so as to keep the total milliwattage (volts X milliamperes) constant. When the power supply (LKB type 3371C) reached maximum setting (ca. 1,200 V), the apparatus was allowed to remain at that level for 16 to 18 h more, during which time the milliamperage dropped to approx. 2.2 mA (2,490 mW). After shutdown, 2-ml fractions were collected from the bottom of the column by pumping water into the top of the column in a closed system to allow accurate control of the rate of fractionation. The pH gradient was read using a Corning model 109 digital pH meter, and a portion of each fraction was diluted 10-fold in phosphate-buffered saline (0.14 M NaCl-0.003 M KCl-0.01 M Na_2HPO_4-KH_2PO_4 [pH 7.4]-1.0 mM $CaCl_2$-0.5 mM $MgCl_2$-0.1% glucose) and titrated on cell monolayers in 1-ounce (0.03-liter) prescription bottles. The recovery of plaque-forming units was in the range of 75 to 100%. All viruses were plaque purified before isoelectrophoresis.

The SPA test (7) was modified (8). In the modified test (Fig. 1) 5 ml of the virion suspension to be tested was put into a cellulose nitrate tube of the Beckman SW 50-1 centrifuge rator opposite a similar tube of a monodisperse suspension of the same virus. After operating the centrifuge just long enough to sediment adhering pairs (and all larger clumps) just below the halfway point in the tube (w^2t=1.23 X 10^{10} for poliovirus or 3.3 X 10^9 for reovirus at 20°C) the rotor was allowed to stop without braking and the upper half of the fluid from each tube was carefully removed for plaque titration. The ratio of the plaque titers of the two supernatant fluids is the fraction of single virions in the unknown suspension. Rate of aggregation tests were made by diluting monodisperse stock virus in acid buffer, for example, at 5 different times and centrifuging them all at once with one unaggregating control sample. By plotting the logarithms of the five plaque titer ratios vs. time a coagulation rate chart was obtained.

In addition to iodometry, ultraviolet absorption spectroscopy was used in the analysis of di-and tribromamine. Ultraviolet absorption spectra of samples from each experiment were taken of a Cary Model 14 spectrophotometer in 10 cm cells. Simultaneous Beer's law equations involving the molar absorptivities of di-and tribroamine at their absorbance maxima of 232 and 258 μm (12) were then solved, showing that each sample was essentially either all dibromamine or all tribromamine, with only minute traces of the other.

Hypochlorous acid (HOCl) at pH 6.0 and hypochlorite ion (OCl^-) at pH 10.0 were prepared from a 1:10 stock solution of Fisher 5% reagent grade sodium hypochlorite solution. The stock was added to 21 L of either pH 6.0 buffer or pH 10.0 hydroxide solution to obtain the desired free chlorine concentration. This concentration was determined immediately prior to, and after a run, by amperometric titration with phenylarsine oxide (PAO) (13).

The pH 6.0 buffer (0.01 M phosphate containing 0.1 M sodium chloride) was prepared from Fisher primary standard monobasic potassium phosphate, Mallinckrodt analytical reagent grade sodium chloride, and deionized glass distilled water. The buffer was made chlorine demand free by adding 2.5 mg/L of chlorine. If after 2 to 4 days the buffer still contained 2.0 to 2.5 mg/L of free chlorine, it was dechlorinated with ultraviolet light and considered chlorine demand free.

The pH 10.0 solution (approximately 10^{-4} M sodium hydroxide) was prepared from Fisher 50% reagent grade sodium hydroxide. Deionized glass-distilled water was made chlorine demand free as described above. The required amount of 50% sodium hydroxide was added to 21 L of this water to bring the pH to 10.0 just prior to the preparation of the OCl^-solution.

Echovirus 1 (Farouk strain) grew well and produced clear plaques on monolayer cultures of HEp-2 cells. It was prepared purified and assayed by the same methods as those used with the poliovirus (mahoney). Physical assay (Particle counting and aggregation analysis) by EM was also the same.

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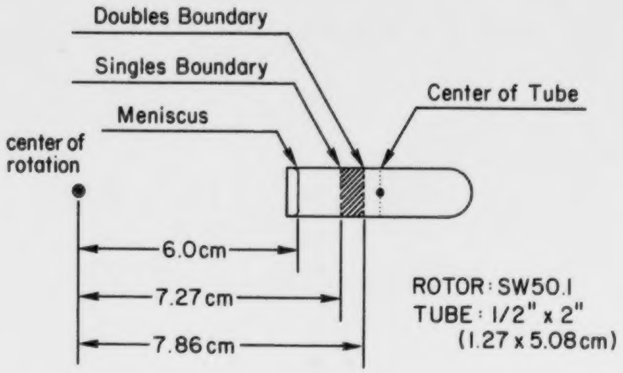

Figure 1. Schematic drawing of centrifuge tube used in the Single Particles Analysis (SPA) of a virion suspension.

Hypobromus acid (HOBr) was produced by adding the stock solution to pH 7 buffer (KH_2PO_4-K_2HPO_4, 0.01 M) just prior to the experiment. As in all experiments, including those involving acetate and NaOH buffers for other bromine species, the buffer was chlorinated to 3-5 mg/L for 4 days and declorinated with an ultraviolet lamp the day before the experiments.

Hypobromite, OBr^-, was also produced by adding stock solution to the buffer immediately before the experiments. One set of experiments was done at pH 10 (0.0001 M NaOH), and the other at pH 11 (0.001 M NaOH). Equilibrium calculations showed that at pH 11, the solution at 4°C was 99% OBr^- and 1% HOBr, while at pH 10, it contained 91% OBr^- and 9% HOBr (9).

Tribromamine, NBr_3, was produced by simultaneously adding equal volumes of OBr^- and NH_4Cl solutions to the pH 7 buffer (KH_2PO_4-K_2HPO_4, 0.01 M). The resulting solutions had an initial molar concentration ratio of conducted within several minutes after preparing the solutions.

Bromine concentrations were determined by the iodometric method (11). Immediately before and after each dynamic inactivation experiment, flow output samples were collected into 250-ml optical red flasks, which already contained 5 ml of 5% potassium iodide. Two milliliters of glacial acetic acid were then added, and the solution was titrated with sodium thiosulfate to an amperometric endpoint, using an applied potential of +200 mV vs. SCE applied to a rotating Pt electrode.

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EXPERIMENTAL RESULTS AND DISCUSSION

SERIES I. INACTIVATION OF POLIOVIRUS (Mahoney) BY BROMINE AND SOME OF ITS COMPOUNDS (4,14)

Purified stock suspension of poliovirus (Mahoney) were stored unfrozen at 4-6°C in the ca 20% sucrose solution, made at pH 7 with phosphate buffer just as they were taken from the density gradient in which they were banded by sedimentation velocity. Figure 2 is a typical picture of such a stock suspension prepared for EM by the KA technique. After allowance is made for coincidence falling of one virion upon another already attached to the collodion film (15) it can be seen that virion aggregation is not over 5%. This is a very critical test but if such a picture of this size showing 2000 virions cannot be shown as evidence for good dispersion, substantial doubt must remain. No centrifuge technique is as critical in the range of 95 to 100% single particles.

Six experiments were made at 2°C, three of which are shown graphically in Fig. 3. All were characterized by an initial linear phase and constant decline in log plaque-forming unit survivor ratio per unit time of exposure. None of these reactions showed any tendency toward delay during the first time interval, which was 4 s. The slopes of all the reactions, ranging from 0.6 to 22 μM bromine concentration, have been plotted in Fig. 4, where it can be seen that increasing bromine concentration does not produce a proportionate increase in reaction rate. The increase becomes progressively less with increasing bromine concentration, indicating a progressive decrease in efficiency.

In several inactivation experiments at bromine concentrations of 3.5 μM or greater, there has been a slight increase in the reaction rate after a substantial period of linearity (see the 22 μM line in Fig. 3). We have not been able to produce this effect regularly. Slopes for Fig. 4 were taken from the linear part of each survival graph.

At 10°C the inactivation rate was constant at 1.0 μM bromine concentration (Fig. 5). There is no indication, in this experiment, that there is any time delay before inactivation begins. However, at higher bromine concentrations the linear part of the graph begins only after the first 4s time interval. It seems unlikely that the initial rate at the higher concentrations could be less than that of the straight line for 1.9 μM bromine, so the lower curves have been drawn tangent to it at zero time. The reaction rates, slopes of the linear parts of the three kinetic experiments, are shown (Fig. 4) to be a linear function of bromine concentration, with an intercept indication zero reaction for zero bromine.

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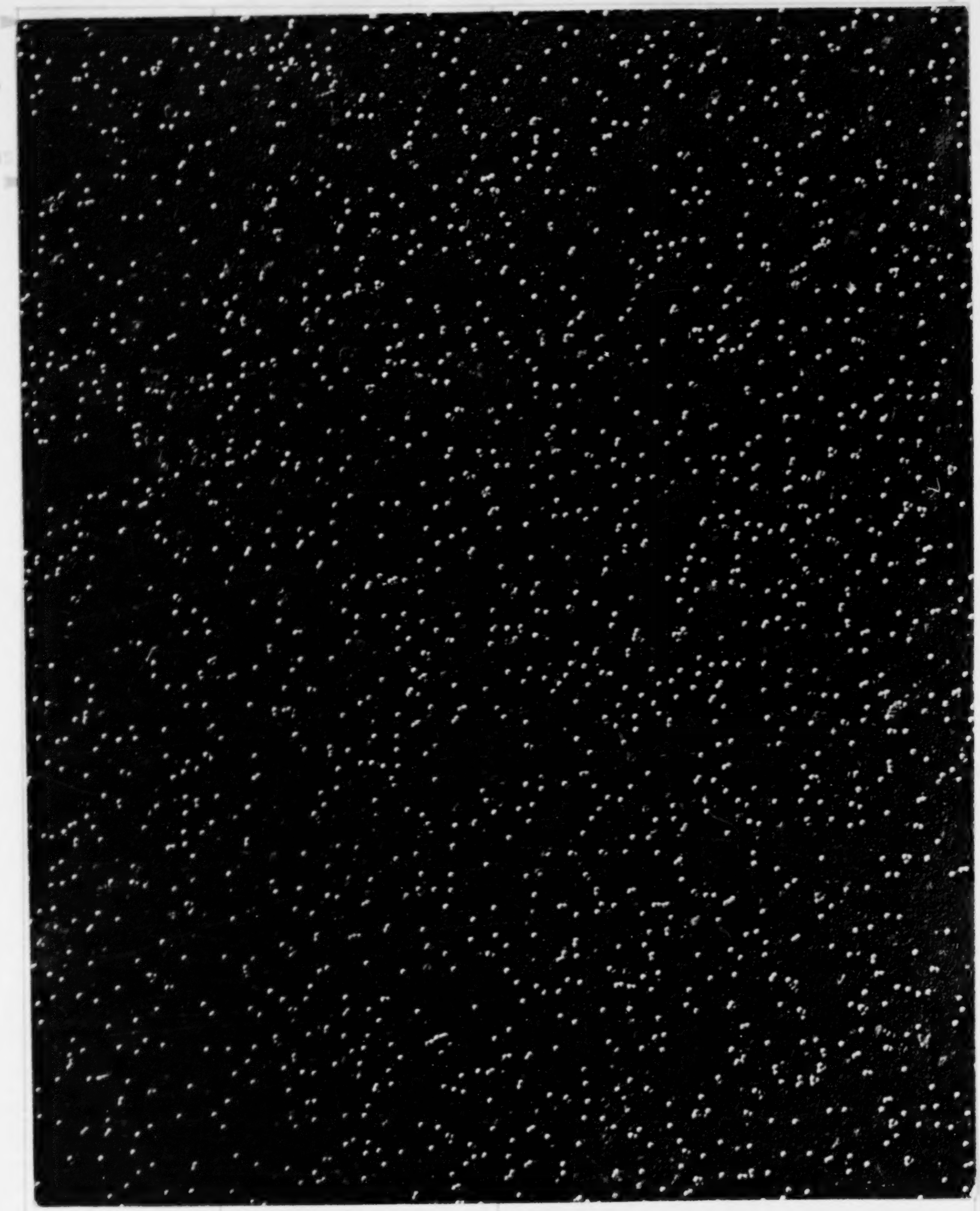

Figure 2. Well dispersed poliovirus, suitable for inactivation rate (IR) experiments.

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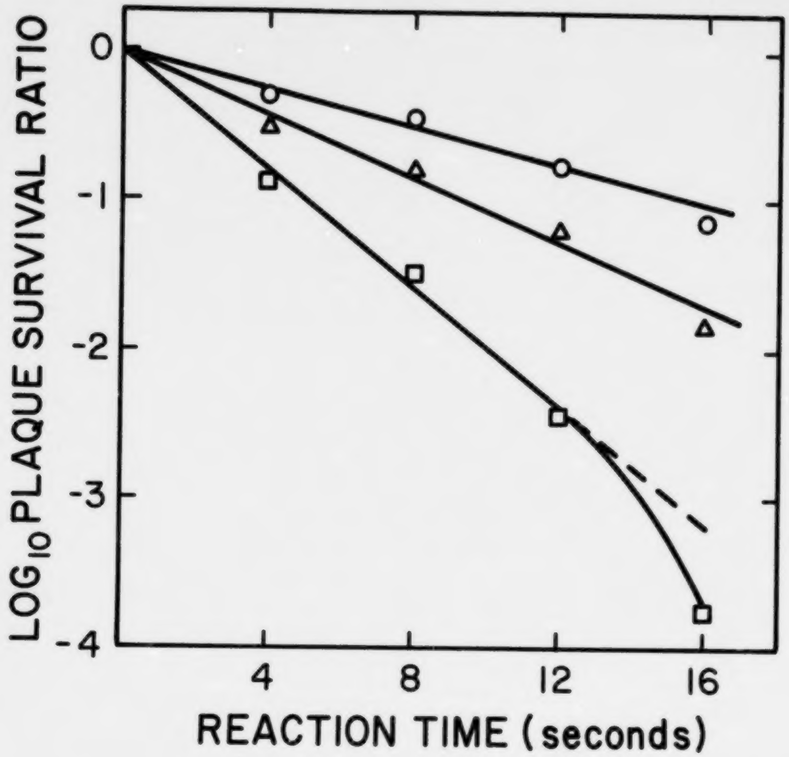

Figure 3. Inactivation kinetics of poliovirus at 2°C, pH 7 by HOBr at 0.6 μM, circles, 2.2 μM, triangles and 22 μM, triangles and 22 μM squares.

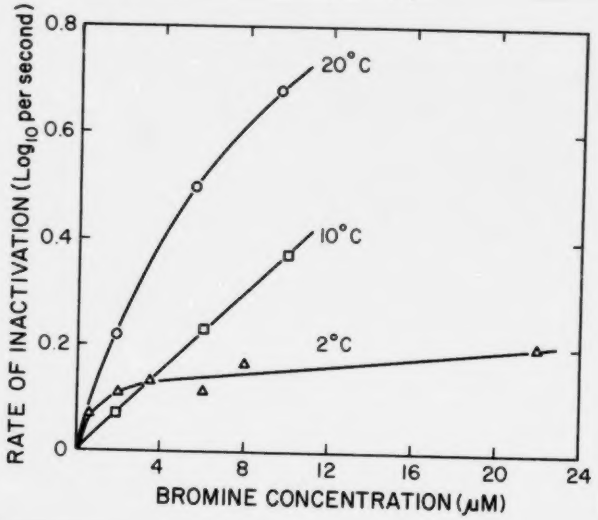

Figure 4. Dependence of inactivation rate on HOBr concentration for poliovirus at pH 7.

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At 20°C the inactivation of the virus proceeded faster at all bromine concentrations than it did at 10°C (Fig. 6). There is some indication in the graph for the 9.5 μM experiment that aggregation is showing its effect at several levels below 10^{-3}. Reaction rates were determined from the straight part of each line.

At 2°C the inactivation of poliovirus by bromine shows no evidence of delay even during the first 4s under conditions leading to 99.9% plaque reduction in 16 s. The logarithm of plaque survival ration decreases linearly with exposure time, as might be expected from the electron microscope data that indicated over 95% of single particles in the preparation. At 10°C only the slowest reaction, the one at 1.9 μM bromine concentration was strictly linear. The faster reactions at higher bromine concentrations showed a slight delay before linear decline rates were established (Fig. 5). Speculation in the past about such delay in the inactivation curve has often invoked aggregation as one of the possible explanations. Now it is clear that the small amount of aggregation in this preparation can have no perceptible effect on the course of the reaction above the 10% survival level. Such a delay may however, be just the time

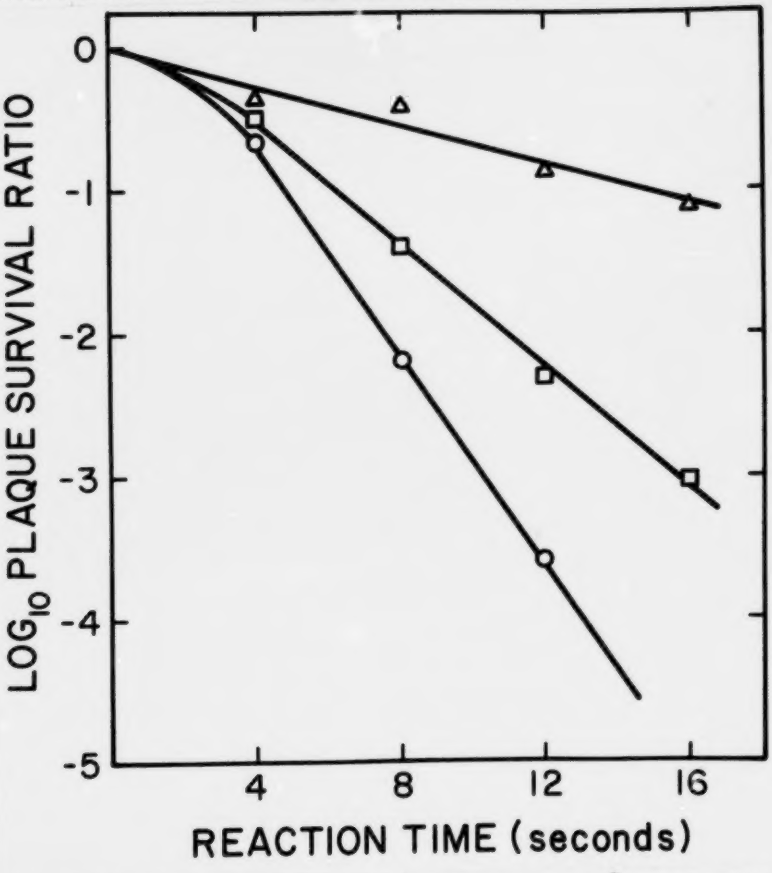

Figure 5. Inactivation kinetics of poliovirus at 10°C and pH7 by HOBr, triangles 1.9 μM, squares 5.9 μM and circles 10 μM.

required for penetration of the bromine to the critical point within the virus capsid.

Inactivation at 20°C was essentially first order over a fivefold range of bromine concentration, and it was substantially faster than that at 10°C, but the summary (Fig. 4) showing the dependence of reaction rates on both bromine concentrations and temperature reveals an unexpected feature. The approach to zero rate at 2°C is quite different from that seen at the two higher temperatures. From bromine concentrations of 22 down to 3.5 μM the rate falls slowly by only 36%. Beyond that point it must plunge steeply to zero or intersect the vertical axis, but not before crossing the 10°C line at 3.5 μM bromine level. This seems to indicate that below 3.5 μM concentration the rate of poliovirus inactivation is greater at 2°C than at 10°C. At this time we have no mechanism to propose that would explain these facts. We are not aware of any significant error in measurement of bromine concentration even at the 1 μM level. However, there is evidence in the work of Weidenkopf (16) with chlorine on poliovirus that at 0°C the inactivation rate depends on chlorine concentration in much the same way as we have seen at 2°C with bromine

Disinfection experiments on poliovirus (Mahoney) with OBr^-were made at pH 10 and 11 at 2°C at concentrations of 2-12 μM (0.32-1.9 mg BR_2/L). Figure 7 shows kinetics of inactivation at pH 11 as the usual semilogarithmic or first-order plot. The inactivation rates were quite rapid initially, but there was a marked decrease in rate with time despite the fact that the virus sample entering the flowing stream of bromine solution was in a monodispersed state. The bottom two curves in Figure 7 (open squares and open circles) show OBr^-experiments performed in the usual manner. The top curve (filled circles) shows an OBr^-inactivation curve of a virus sample that was pretreated by centrifugation in a Beckman SW 50.1 rotor at 30,000 rpm for 25 min. at 20°C. This treatment removed all aggregates of size larger than pairs and left monodispersed virus in the top 3 ml of the tube. Thus, if any aggregates were present in the virus preparation that were not seen by electron microscopy, they would have been removed by the centrifugation. However, this OBr^-inactivation curve gave the same shape and concentration dependence as the nonprecentrifuged virus, indicating that virus aggregation played no part in causing the decrease in slope with time.

Table 2 shows the initial reaction rates at pH 11 for each of the three curves shown in Figure 7. These values were estimated by taking the first derivative of a polynomial fitted to the points as a curve. Since all curves gave zero inactivation at zero time, the first derivative yielded an estimate of the slope of the curve at time zero, and hence an estimate of the initial rate of inactivation. These calculations were performed in this manner since there was no linear portion of the curve to use as a basis of comparision as there was with HOBr.

Figure 8 shows the kinetics of inactivation of poliovirus with OBr^-at pH 10. As at pH 11, all inactivation rates decreased with time despite the use of nonaggregated virus; however, the rates of inactivation were less

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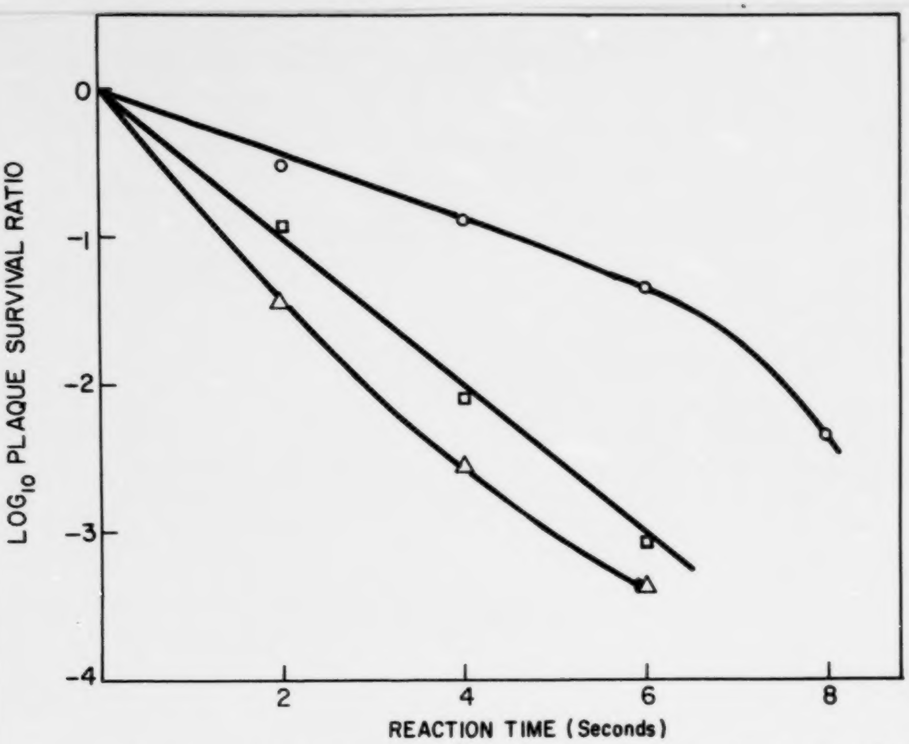

Figure 6. Inactivation of poliovirus at 20°C and pH7 by HOBr, circles 1.9 μM, squares 5.5 μM and triangles 9.5 μM.

than at pH 11. Table 2 gives initial reaction rates at pH 10 which are in all cases less than 2.0 $\log_{10}$/s.

Table 2 also demonstrates a saturation effect with respect to OBr^- at pH 10, similar to that observed with HOBr at pH 7 and 2°C (4). Although the OBr^- concentration at pH 10 doubled from 6 to 12 μM, no increase was seen in the initial rate of inactivation. The pH 11 data do not show this effect since an increase of OBr^- from 5.5 to 9.6 μM (1.75-fold) yielded an increase of initial rate from -2.1 to -3.85 $\log_{10}$/s (1.83-fold). This linear increase in rate at pH 11 was similar to that at pH 7 with HOBr at higher temperatures of 10 and 20°C.

The inactivation power of Br_2 was tested at pH 5.0, under which conditions aggregation of the virus was massive. Therefore, to prevent aggregation of the virus, 0.3 M NaCl was added to the buffer solution as previously described (7). Figure 9 shows the kinetics of inactivation of poliovirus with Br_2/L). The plots of these results were linear with time and showed no tendency to curve as did the OBr^- data. The reaction rates were -0.8, -1.81, and -2.56 $\log_{10}$/s, respectively, for the three concentrations of Br_2 tested. Although slower than the initial rates for OBr^-, they are approximately ten times greater than for HOBr. When the rate of

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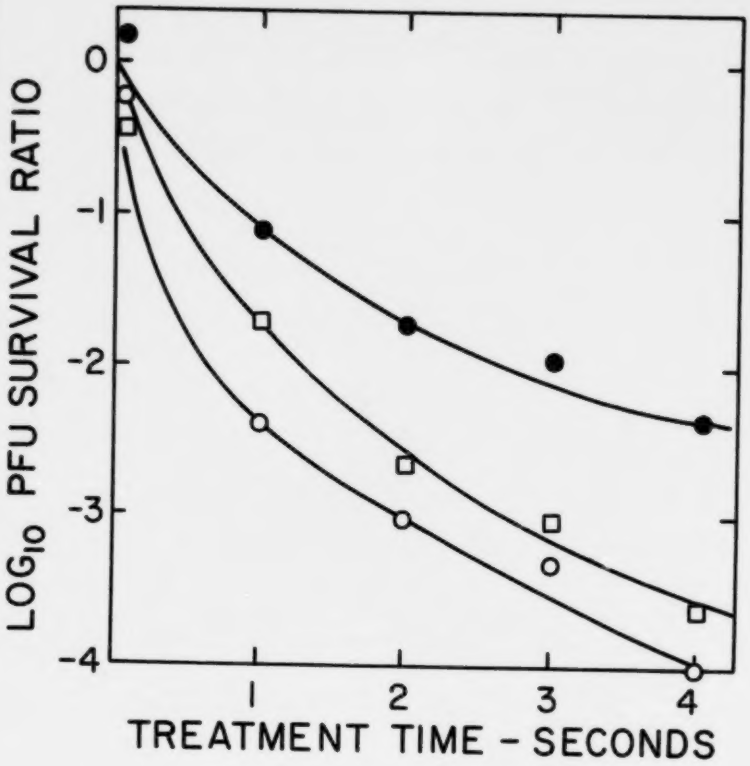

Figure 7. Kinetics of inactivation of poliovirus at 2°C and pH 11 by hypobromite ion (OBr^-), filled circles 4.3 μM, squares 5.5 μM and open circles 9.6 μM.

inactivation was plotted against the bromine concentration (Figure 10), a generally linear increase in rate with increasing concentration was found.

To demonstrate the effect of aggregation on the inactivation of virus, a series of experiments was performed with Br_2 at pH 5 in the absence of added NaCl. Two such experiments at 12.9 and 19.7 mM (2.06 and 3.15 mg Br_2/L) are shown in Figure 11 (open triangles and squares) together with one experiment at 21.6 μM Br_2 (3.46 mg Br_2/L) in the presence of 0.3 M NaCl (open circles) for comparison. The virus sample was placed at pH 5 in acetate buffer 1 h before injection into the flowing stream to allow aggregation to occur. Under these conditions, the initial inactivation rate was similar to that of a dispersed virus because of the presence of residual single particles, but the rate of inactivation decreased markedly with time due to the presence of viral aggregation. The data points were scattered to a much greater degree than those of the experiments without viral aggregation; this appears to be a typical feature of experiments with aggregated virus.

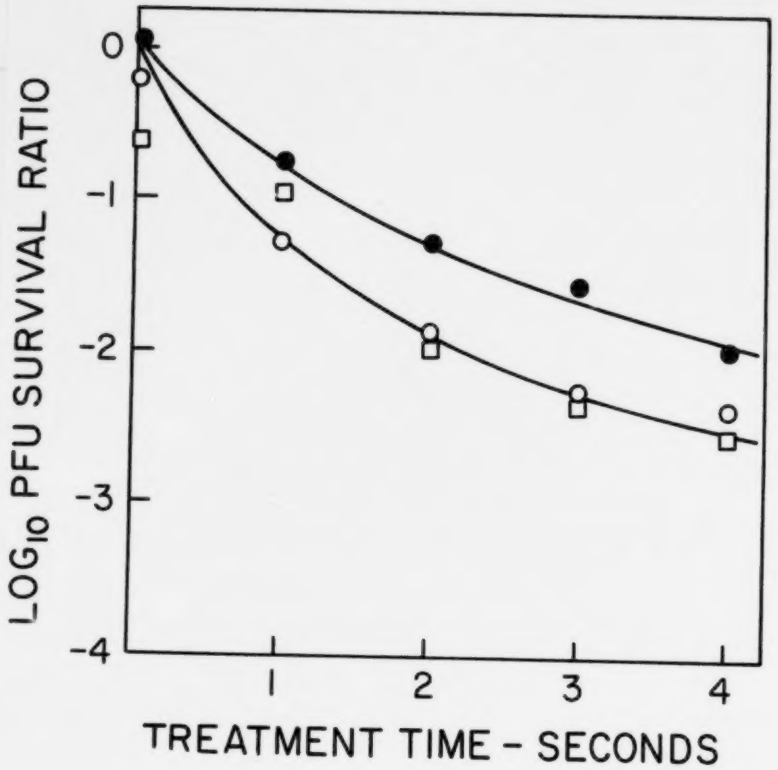

Figure 8. Kinetics of inactivation of poliovirus at 4°C and pH10 by OBr^-, filled circles 2 μM, squares 6 μM and open circles 12 μM.

Kinetics of inactivation of poliovirus with NBr_3 at 4°C are shown in Figure 12 and the relation between rate of inactivation and NBr_3 concentration is shown in Figure 13. At the lowest concentration tested, 3.2 μM NBr_3 (1.5) mg Br_2/L, there was a lag of several seconds in the kinetic curve before a linear phase of first-order inactivation was reached. Higher concentrations of NBr_3 gave strictly linear plots without the appearance of a shoulder, but there was a marked tendency toward saturation (Figure 13) above a concentration of 25 μM NBr_3 (12 mg Br_2/L). No further increase in reaction rate was observed at higher concentrations of Br_2.

TABLE 2. Calculated values for Initial Rates of Inactivation of Poliovirus by Hypobromite (OBr) at pH 11 and 10

	Initial rates of inactivation		
pH11:			
Concentration of OBr^- (μM)	4.25	5.5	9.6
Initial rate[a] of inactivation ($\log^{10}$/S)	-1.28	-2.1	-3.85
pH 10:			
Concentration of OBR^- (μM)	2.0	6.0	12.0
Initial rate[a] of inactivation ($\log^{10}$/S)	-0.95	-1.9	-1.95

[a] Calculated from the first derivative of the equation $y= ax^4 + bx^3 + cx^2 + dx$ at 0 time

Dibromamine was the least effective compound of bromine tested. The rate or reaction was slow enough that a modification was necessary in the continuous flow apparatus to take samples at intervals of minutes rather than seconds. The modification consisted of a flowing stream of 15 s length terminated by one sample port where a sample could be taken at this point if desired. As soon as the virus-containing buffered bromine solution came to the end of the tube, approximately 60 ml (about 2 s in time) of solution was caught in a beaker held in an ice bath to maintain the temperature of 4°C. Samples were taken from this beaker at the appropriate intervals of time. The data from four experiments are plotted in Figure 14. At the highest concentration tested the results were essentially linear, while at the lower concentrations the results were very strongly curved in spite of the absence of any significant aggregation.

A graphical resume of all these data is shown in Figure 15. All of the data accumulated in this series were obtained at 2-4°C as indicated.

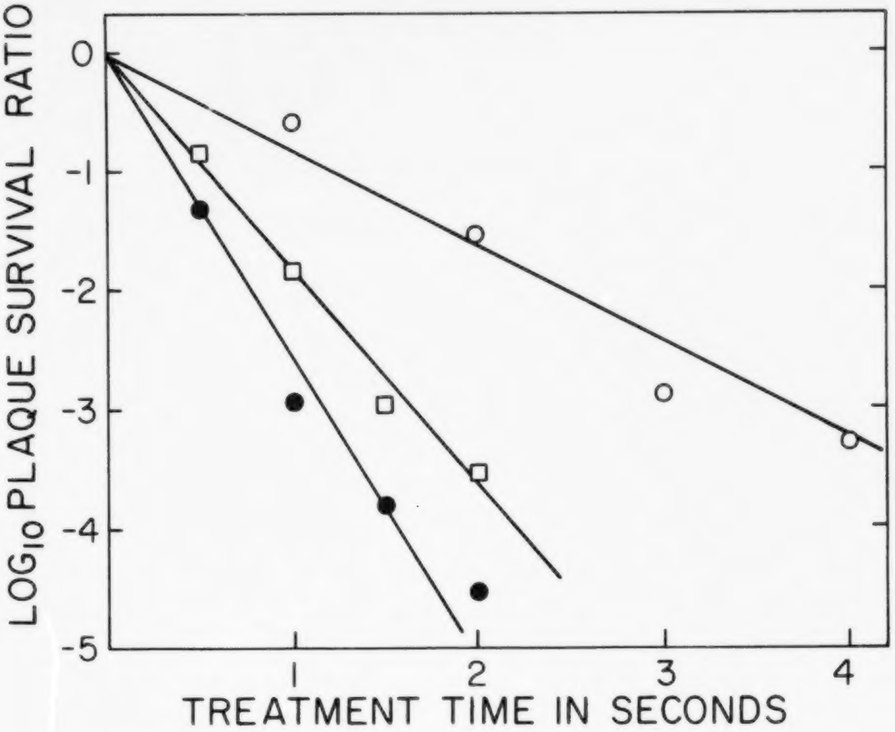

Figure 9. Kinetics of inactivation of poliovirus at 4°C and pH5 in the presence of 0.3 M NaCl by Br_2, open circles 4.7 μM, squares 12.9 μM and filled circles 21.6 μM.

The four different compunds of bromine tested here for their destruction of the infectivity of poliovirus are very different in potency. In comparing these it is helpful to use molar concentrations; however, it is difficult to make an equally uniform statement of reaction rates. For those that produce a linear decline in log PFU with time (Br_2, NBr_3, and HOBr), rates can be expressed simply as the slope of the line, as $\log_{10}$/s. With OBr^-, however, there is an initially rapid reaction whos rate decreases with time. For these one may select the initial rate for comparison purposes, or one may choose to compare times required to reduce the starting titer of the virus to some selected value such as 10 or 1%, or 1/3 as advocated by Morris (17). Of course, the latter procedure leads to very different potency ratios depending on the end point level selected, particularly when one of the agents compared produces a curve (on the logarithmic scale) and the other, a straight line. We have elected to calculate the approximate slope at zero time by using a polynomial equation

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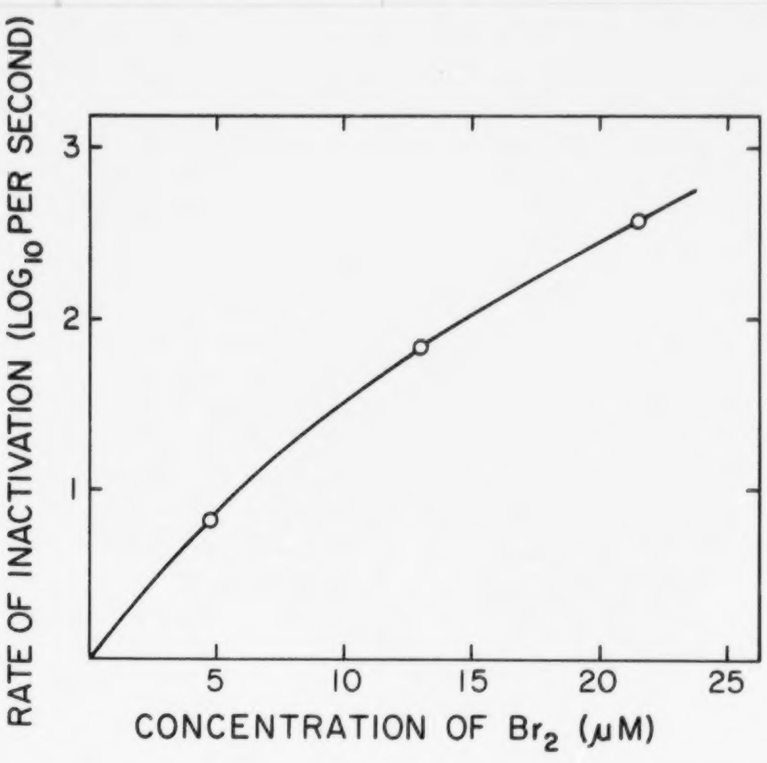

Figure 10. Dependence of inactivation rate of poliovirus on concentration of molecular bromine (BR_2).

to estimate the initial rate of the OBr^- inactivation data because of its simplicity and applicability. Obviously, no system of calculations can yield an accurate initial rate at zero time in the absence of numerous data points at or near this time point, which are difficult to take in small fractions of the first second. Hence, there is no single number, ratio, or other statistic that can be devised to summarize the graphical data and give an overall statement of the relative potency of all five of the common bromine compounds in water.

Low temperatures (2-4°C) were chosen for this work because at higher temperatures some of the reactions would have been too rapid to follow. The different pH values required for the different bromine compounds are well within the limits of stability of the poliovirus infectivity. Loss of titer, even at pH 11, is negligible in the short time required for these experiments.

The initial rate for OBr^-, the linear rates for Br_2 NBr_3 and HOBr, and the initial rate of $NHBr_2$, all at 10 μM concentration, are 3.85, 1.55, 0.15, 0.16, and 0.013 $\log_{10}/s$, respectively. When this work was done there were no complete data for direct comparison with a similar set of chlorine compounds. Some points of comparison can be made; however, the state of

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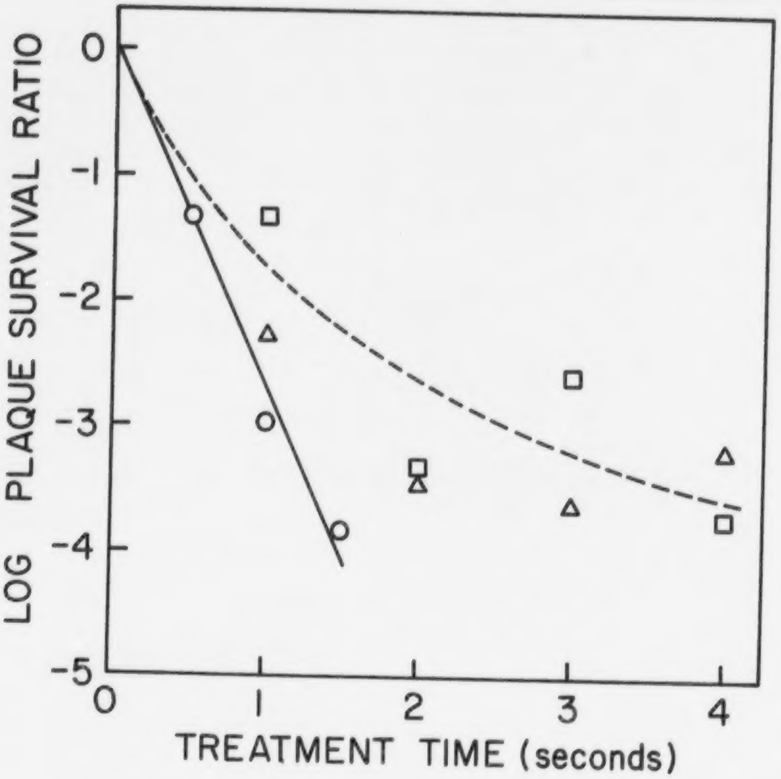

Figure 11. Kinetics of inactivation of poliovirus at 4°C and pH5 by 12.9 μM, Br_2, triangles, 19.7 μM Br_2, squares and by 21.6 μM Br_2 in the presence of 0.3 M NaCl, circles.

virion aggregation has often been ignored in earlier experiments, and if data were taken on the time to reach a given decrement in virus titer the presence of aggregates may make reaction rates look low by comparison with data presented here. Generally speaking, it appears that bromine inactivates poliovirus faster than equimolar concentrations of either chlorine or iodine. Four publications of carefully controlled laboratory experiments have been chosen to illustrate this point. Weidenkopf (16) found that type 1 Mahoney poliovirus was inactivated at the rate of 1.23 $\log_{10}$ units per minute when 5.5 μM (0.39 mg Cl_2/L) free chlorine (HOCl) was present at 0°C, pH6. Other concentrations in the range 1.43-23.6 μM were tested, and a graph of these values was plotted by us (4), which shows the same shape of curve as that for NBr_3 in this work. Although reaction rates increase with concentration, both bromine and chlorine appeared to lose

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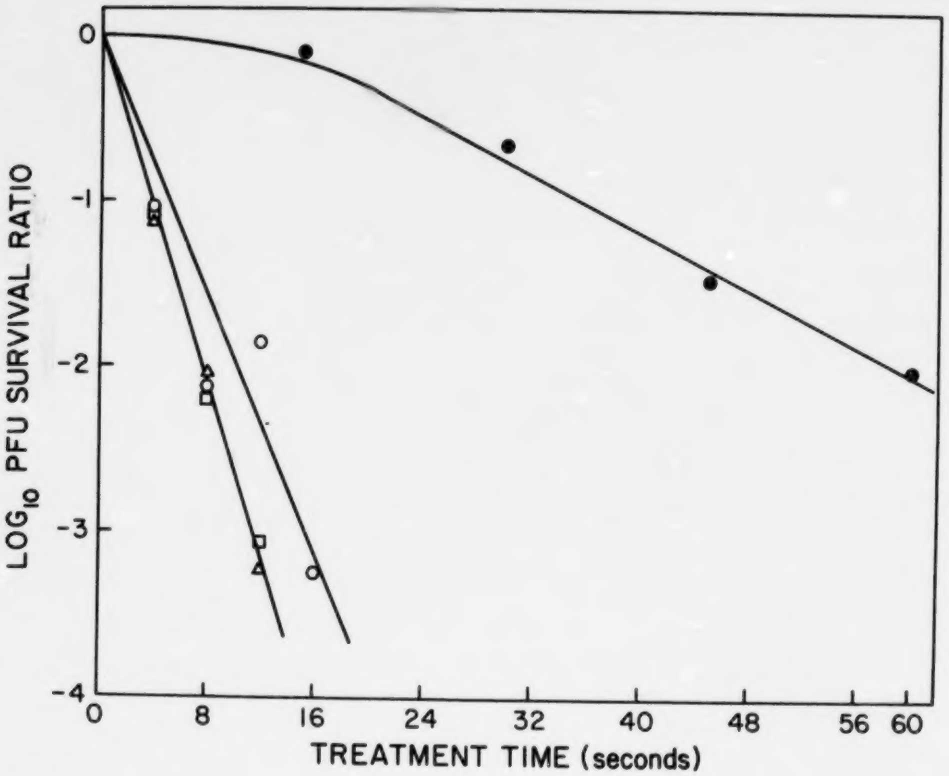

Figure 12. Kinetics of inactivation of poliovirus at 4°C by Tribromamine (NBr_3), 3.2 μM, filled circles, 12 μM, open circles, 27 μM, squares and 49 μM, triangles.

efficiency with increased concentration. Figure 12 shows that with 3.2 μM NBr_3 (154 mg Br_2/L) the virus was degraded 0.044 $\log_{10}$/s or 2.64 $\log_{10}$/min in the linear portion of the curve. This is about 5.3 times faster for similar μM concentrations than that given by Weidenkopf for chlorine. At 14 μM, where our data shows NBr_3 reducing infectivity of 0.23 $\log_{10}$/s, Weidenkopf found 1.8 $\log_{10}$/min or 0.03 $\log_{10}$/s. Here the ratio is 7.6 times faster for the same μM concentration of tribromamine.

Scarpino et al. (18) found that for 1 mg/L free chlorine (HOCl), which is 14 μM, there was a decrease in titer of 2 $\log_{10}$ in 126s, or 0.95 $\log_{10}$/min, roughly 1/2 the rate of Weidenkopf even though the temperature was 5°C higher.

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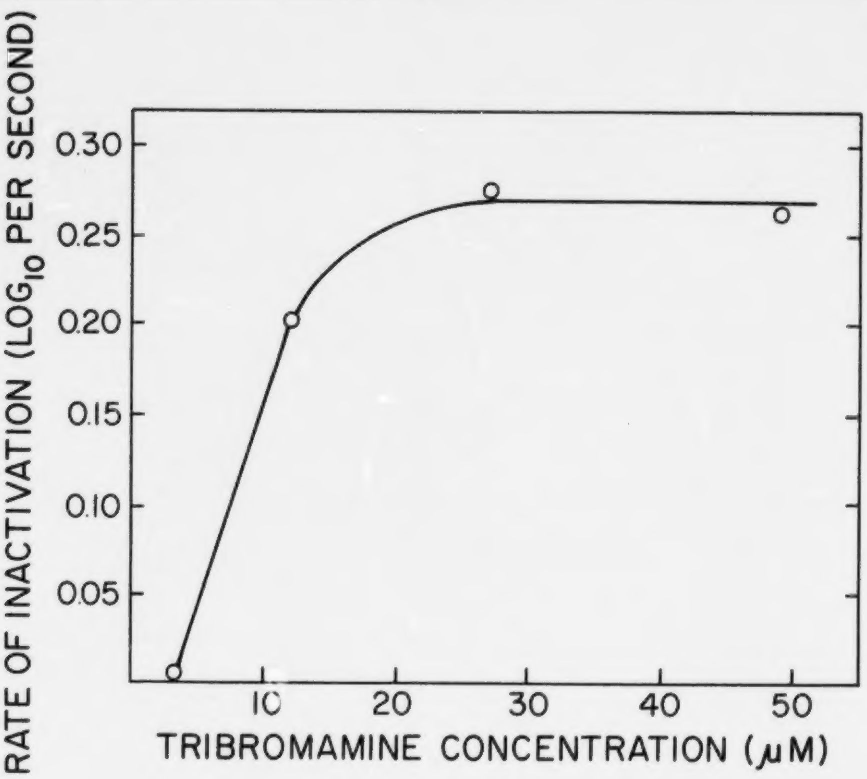

Figure 13. Dependence of inactivation rate of poliovirus at 4°C on concentration of NBr_3.

Symons and Hoff (19), exposing poliovirus at pH 6, 5°C to 1.5 mg Cl_2/L, obtained a curve, the initial slope of which we have calculated to be 0.21 log/s. This rate is nearly the same as our rate for HOBr and NBr_3. This high rate was not maintained however. At 1 min it had declined to $0.033/\log_{10}/s$ or 2 $\log_{10}$/min.

Berg et al (20), with 20 μM of elemental iodine (5.08 mg I_2/L) at pH 6 and 15°C, obtained a two $\log_{10}$ decrease in poliovirus titer in 58 min. A substantial lag period was observed before the reaction entered the linear semilog phase, but even in the linear phase the inactivation of the virus by iodine is less than 10% of the rate for bromine.

The experimental facts gathered here reveal a phenomenon heretofore obscured. When the inactivation rate decreases during the course of a disinfection experiment with purified virus, it is usually attributed to virion aggregation, assuming that genetic heterogeneity and depletion of the disinfecting agent are excluded. Here we have seen with two bromine

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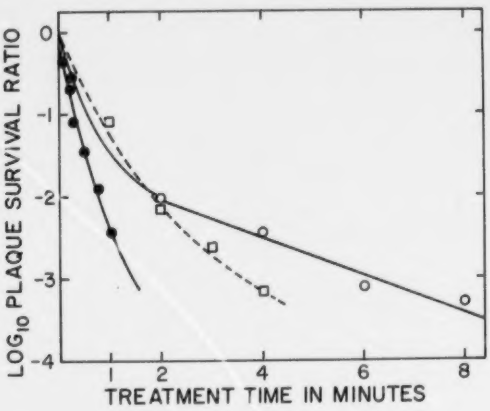

Figure 14. Kinetics of inactivation of poliovirus at 4°C by dibromamine ($NHBR_2$), open circles 2.9 μM, squares 13.8 μM and filled circles 39 μM.

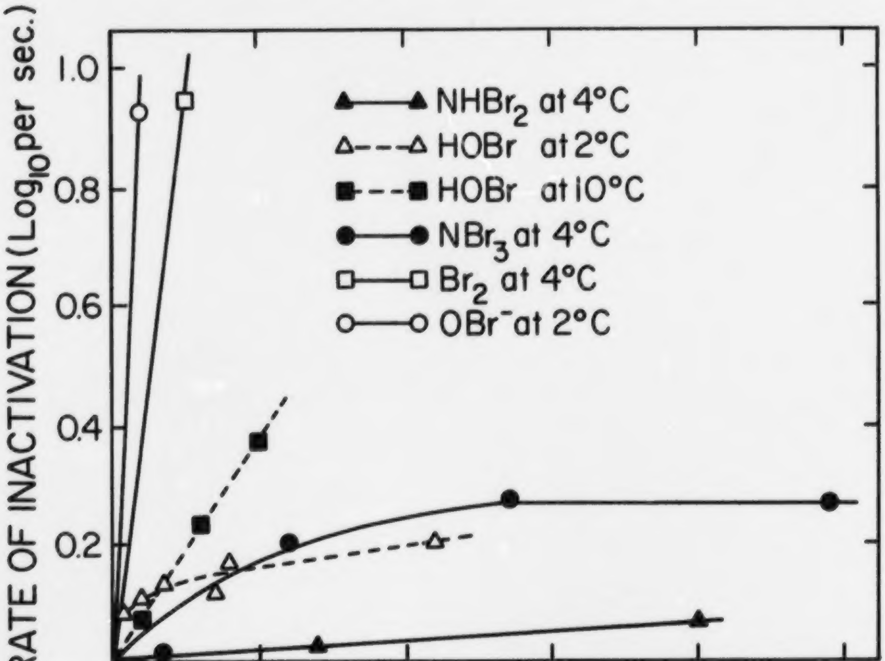

Figure 15. Dependence of inactivation rate of poliovirus on concentration of several bromine agents.

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compounds (Br_2 and NBr_3) and with HOBr, linear reaction rates extending three and four factors of 10 when aggregation is very low or absent. The same virus, however, shows a declining reaction rate when treated with two other bromine compounds (OBr^- and $NHBr_2$). This might be interpreted to mean that a small but significant amount of aggregation remains, and the two classes of bromine compounds respond to it in very different degrees. But it seems more likely that the influence of aggregation has been effectively removed, and different modes of action of these two groups of compounds on poliovirus are now clearly revealed. The next step must be to determine the nature of these reactions, and hence the manner of their difference.

Although no studies have been reported on the viricidal activity of OBr^-, Taylor and Johnson (21) studied Br_2, HOBr, and NBr_3 and suggested that OBr^- should resemble OCl^- in activity. Preliminary experiments with OBr^- and poliovirus revealed rapid inactivation kinetics, and the results given herein have been reproduced several times. Thus, the activity appears to be consistently rapid. However, we have not yet been able to formulate a mechanism for the rapid activity of OBr^- compared to the much slower activity of OCl^-, and it will be necessary to study this phenomenon further before any definite mechanism can be revealed.

SERIES II. INACTIVATION BY CHLORINE OF POLIOVIRUS (MAHONEY) IN WATER (22)

The inactivation of poliovirus single particles by HOCl at pH 6 was observed at four different temperatures, 2, 10, 20 and 30°C, and the results are shown in Figures 16, 17. 18, and 19, respectively. The concentrations used in these experiments were in the range 1.4 to 40 μM (0.1 to 2.8 mg/L). Because the virus suspension used in these experiments could not be definitely shown to be free of aggregation at a level below 10^{-3} of the particle count (see below), all experiments were terminated at a survival level no greater than 10^{-4}. With single particles at the chlorine concentrations used (above), the 10^{-4} survival level was reached in times less than 1 min. It was therefore essential to utilize the fast-flow apparatus (1) to be able to take samples at intervals as short as 1 s.

The kinetic time curves as shown in Figures 16-19 show a generally consistent pattern of inactivation regardless of temperature. At the lower concentrations of 1-2 μM (0.07-0.14 mg/L as Cl_2) the reaction appeared to be linear, although in the times taken the survival level reached was less than 10^{-1}. At higher concentrations of 20-40 μM (1.4-2.8 mg/L as Cl^2), and especially at the highest temperature of 30°C, there was an initial lag on the inactivation curve which persisted a few seconds to almost -0.6 log at most, but after this point, the major portion of each curve was linear in character. These results are similar to previous work with single particles of poliovirus and inactivation by HOBr at 10 and 20°C.

In order to compare reaction rates of chlorine at the four temperatures, we have taken the slope of the straight-line portion of the kinetic curves, calculated by the least-squares methods, as a measure of the rate of reaction and this has been plotted against the chlorine concentration in a manner similar to that for HOBr and other bromine compounds. These curves are shown in Figure 20. At all four temperatures, the points, when plotted in this manner, fell on a biphasic curve, similar to HOBr at 2°C, and to NBr_3 at 4°C. It can be seen from these curves that (i) an increase in temperature resulted in an increase in reaction rate, and (ii) a sharp decrease in slope was observed in the first section of the curves. However, within the second portion of the biphasic curve there was still a slow increase in activation rates with increasing HOCl concentration. This was most pronounced at 20 and 30°C, while at 2°C, an increase in HOCl from 20 to 40 μM (1.4 to 2.8 mg/L as Cl_2) yielded only an increase in rate from 0.165 log/s to 0.2 log/s. These results show that low concentrations of HOCl are the most efficient for inactivation of the virus.

The results of four experiments with hypochlorite ion (OCl^-) at pH 10 are shown in Figure 21. The reaction rates under these conditions were

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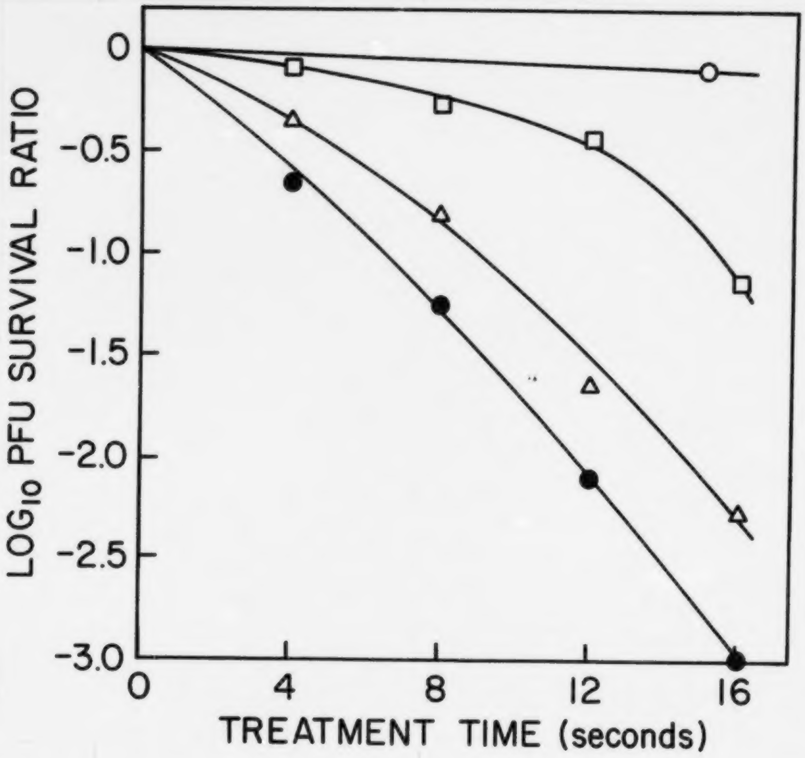

Figure 16. Inactivation of poliovirus single particles at 2°C by HOCl, 1.4 μM circles, 10 μM squares, 22 μM triangles and 40 μM filled circles.

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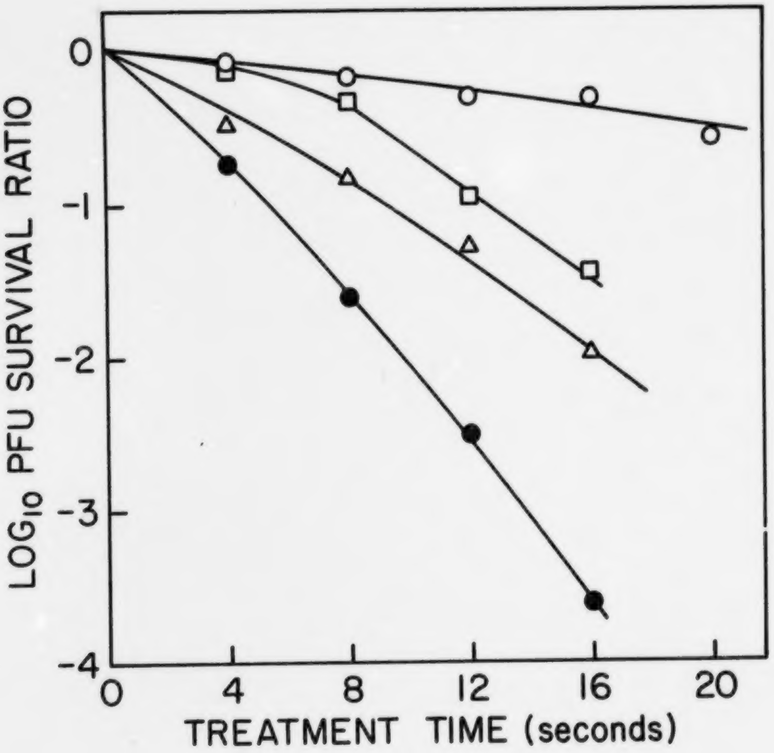

Figure 17. Inactivation of poliovirus at 10°C by HOCl, 1.5 μM circles, 11 μM squares, 20 μM triangles and 41 μM filled circles.

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considerable slower than HOCl at Ph 6, so much so that it was necessary to take samples for virus titration at intervals of 0.5-1.0 min in order to obtain noticeable inactivation. All tests were made at 20°C. The overall shape of the kinetic inactivation curve appeared to be OCl^- concentration dependent. The lowest concentration of 3.5 μM (0.25 mg/L) yielded a curve having an initial lag of about 1.0 min, then became linear out to -1.8 log inactivation in 6 min with an inactivation rate of -0.34 log/min, or -0.0057 log/s. At 7.5 μM OCl^-(0.53 mg/L) the initial activation was linear with an inactivation rate of 0.9 log/min, or -0.015 log/s. After 2.0 min, the reaction rate appeared to be reduced. At the highest concentrations tested, 15 and 30 μM (1.07 and 2.14 mg/L) the same trend was observed. After a 2 minute linear reaction, the rate dropped with increasing time resulting in a retarded dieaway. The initial rates for these two concentrations were -1.78 and -1.95 log/min. for 15 and 30 μM OCl^- (1.07 and 2.14 mg/L), respectively. Thus, there appeared to be no significant increase in the initial rate of inactivation with increasing OCl^- concentration from 15 to 30 μM OCl^-. These results are similar to those reported for OBr^- at pH 10, and they suggest that the mechanism of inactivation by OCl^- is different from that by HOCl. In addition, the reaction rate of OCl^- was roughly one tenth that of HOCl. For example, at 22 μM HOCl (1.6 mg/L) at pH 6 the survival ratio was reduced to 10^{-2} in 7.5 s; with OCl^- at 15-30 μM at pH 10 it took 70 s to produce the same result, even though the HOCl concentration at pH 10 was only 0.06-0.12 μM (0.004-008 mg/L).

In our previous report (1) we presented data showing the degradation of reovirus particles after treatment with HOBr at pH 7. This treatment caused a loss of viral RNA and some destruction of coat protein but the number of degraded particles was the same as the number of untreated particles in the controls. This type of physical change appeared to be unique and hence it was of considerable importance to examine by EM the effects of HOCl on poliovirus. This was not possible until the technique described here in materials and methods was developed. Because of the small size of the poliomyelitis virus particles, all attempts to esamine them after HOCl treatment in fluid suspension failed. The degraded particles would not attach to the collodion film so it was necessary to deposit untreated virions on the film and perform the HOCl treatment in situ. It is not necessary to reproduce the pictures here because the results can be readily described. Most of the particles of the untreated virus exclude the PTA stain although a few imperfect ones are present. After 15 seconds exposure to 5 μM HOCl very few more virions appeared damaged sufficiently to permit penetration of the PTA. This treatment should have reduced the PFU survivial ratio below one percent. After 15 seconds at 15 μM HOCl there was as many as one third of the particles admitting PTA and many showing more advanced degradation but many still appeared to be intact in spite of an estimated survival ratio of 10^{-4}. Exposure of the virus to 30 μM HOCl for 15 seconds produced mostly debris difficult to identify as virus although a few intact particles remained (estimated survival ratio 10^{-6}).

Experiments in this laboratory on the inactivation of monodispersed preparations of poliovirus with several different active forms of bromine

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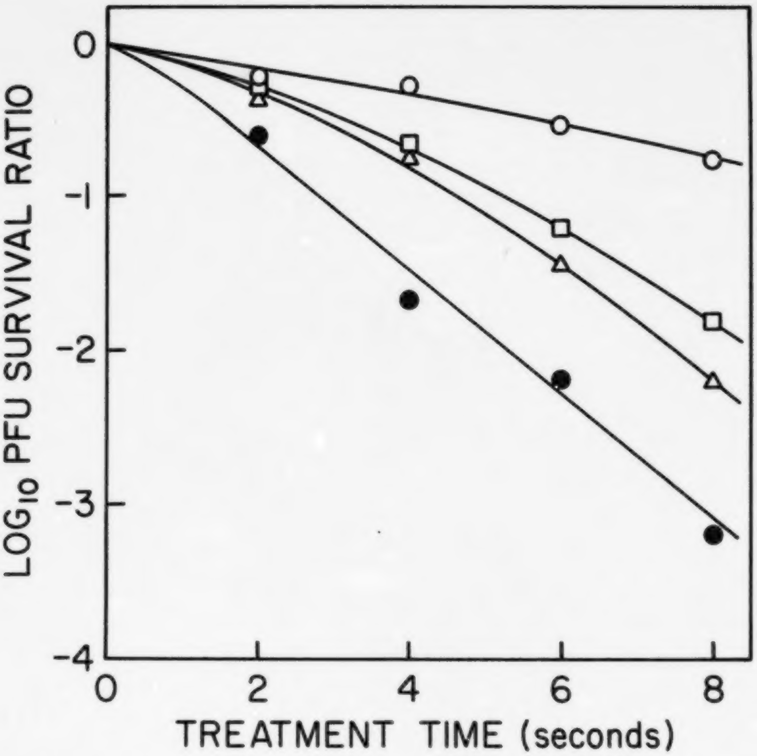

Figure 18. Inactivation of poliovirus at 20°C by HOCl, 2.2 μM circles 11 μM squares, 22 μM triangles and 35 μM filled circles.

have revealed inactivation rates much greater than those reported in the literature for chlorine. Now that similar experiments with monodispersed preparations of virus have been made with chlorine, it appears that the difference between HOBr and HOCl is not as great as previously beleived. For example, from Figure 3, it can be seen that 22 μM HOBr at 2°C reduced the plaque level to 10^{-3} in 16s. It took 40 μM HOCl to produce essentially the same results (Figure 16). Both of these rates are greater than those reported by Scarpino et al. (18), Weidenkopf (16), or Symons and Hoff (19). It is possible to draw roughly a tangent line to the semi-log inactivation curve of Symons and Holf at zero time, which reveals an initial rate for their virus preparations approximately the same as for our poliovirus preparations given in Figure 16. The fact that their reaction rate decreased with time is probably evidence that surviving PFU were virus aggregates of many sizes. If their virus rate contained a high proportion of single particles, the initial rate would be expected to equal that of an all-singles preparation, and this was apparently the case.

The effects of chlorine and bromine are most conspicuously different when OCl^- and OBr^- are compared with each other, and with HOCl and HOBr. Hypobromite ion is more effective than HOBr, whereas HOCl is far more efficientthan OCl^-. The relative rates appear to be in the order: $OBr^- >$

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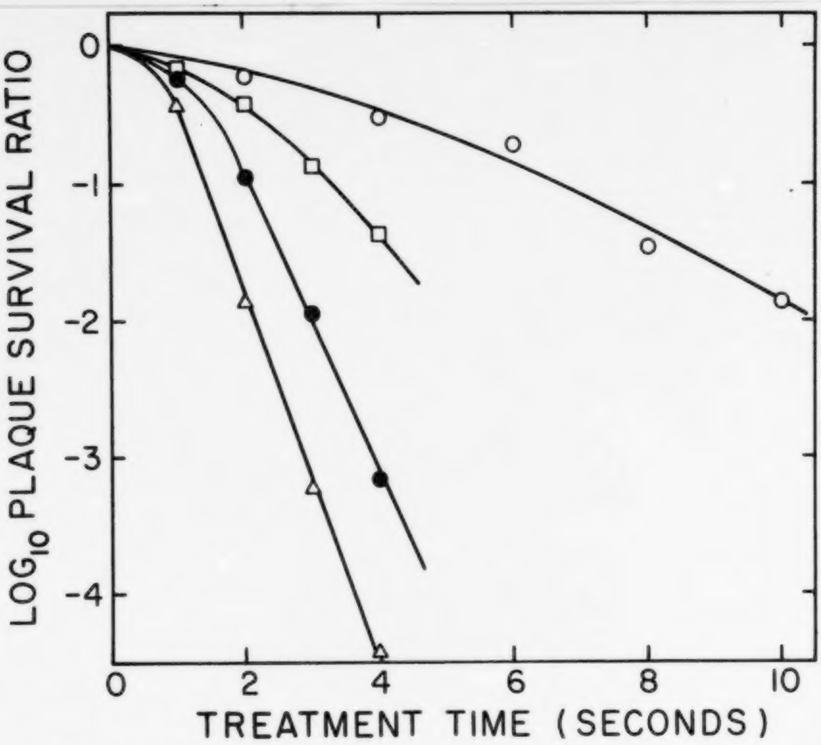

Figure 19. Inactivation of poliovirus at 30°C by HOCl, 1.4 μM circles, 10 μM squares, 20 μM filled circles and 42 μM triangles.

HOBr >HOCl >OCl$^-$. These findings are at variance with those Scarpino et al. (18), but are similar in one respect to the data of Weidenkopf (16).

The data from Scarpino et al. (18), using the Mahoney strain of poliovirus type 1, show OCl$^-$ inactivating more rapidly than HOCl, the reverse of the results reported here. For instance, with respect to HOCl, Scarpino et al. reported that at 14 μM (1 mg/L) their time to inactivate to -2.0 (99%) at pH 6, 5°C, in phosphate butter would have been 117s. Under quite similar circumstances, although at 2°C, Figure 5 shows that at 14 μM our rate would have been 0.11 log/s, or 18.2s to reach -2.0 log. This is in the ratio of 18.2:117; our rate is therefore about 6.4X faster than that of Scarpino et al. Conversely, at 7 μM (0.5 mg/L) of OCl$^-$, Scarpino's rate to reach -2 log was 50 s. Our time to reach -2 log at 7.5 M OCl$^-$ (0.53 mg/L) was 153 s. Thus, the rate of Scarpino et al. is approximately 3.1X faster than ours for OCl$^-$.

This difference may be related in some as yet obscure way to the fact that Scarpino et al. have treated their virus at pH 10 in borate buffer containing 0.05 M KCl. We were not able to obtain an aggregation-free suspension of virus under these conditions. Even in the presence of the KCl, borate buffered virus sedimented in the ultracentrifuge by the Single Particle Analysis test faster than single poliovirions; we have avoided for

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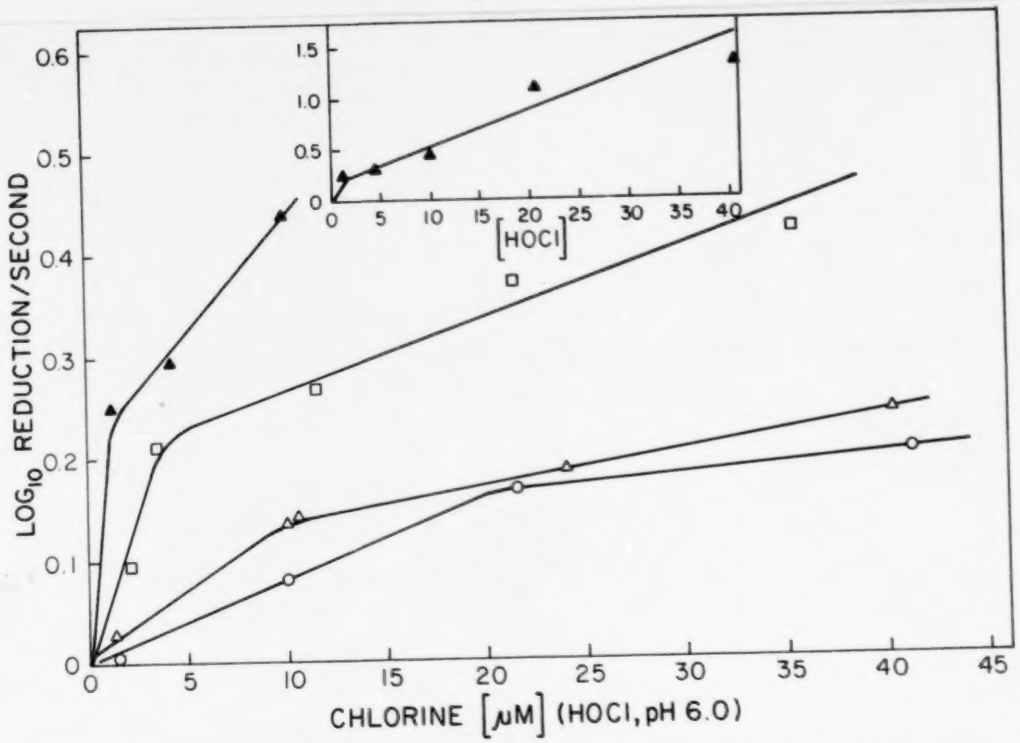

Figure 20. Dependence of inactivation rate of poliovirus on HOCl concentration at 2°C (circles), 10°C (triangles), 20°C (circles), 10°C (triangles), 20°C (squares) and 30°C (filled triangles).

the present any unexpected complications by adjusting the chlorine solution to pH 10 with Na OH. The pH remained constant during the experiment and there was no evidence of aggregation.

In contrast to the data of Scarpino et al., the results obtained by Kott et al. (23) indicated that OCl^- was less effective as a disinfecting agent than HOCl, consistent with our data reported here.

With the data from the paper by Weidenkopf (16), there is not as marked a difference between his results and ours presented here, although his high pH experiments show one similarity to ours at pH 10. Weidenkopf shows 141 s to reach -2 log at 5.5 M HOCl (0.39 mg/L) at pH 6 in phosphate buffer using Mahoney poliovirus. Our data show a rate of 0.042 log/s at 5.5 M, or 47 s to reach -2 log. The ratio here is 47:141; our data are therefore show about a three-fold faster rate. Weidenkopf did not perform any tests at pH 10 as in our data here, but the results of his experiments performed at pH 6,7, and 8.5 showed a definite reduction in reaction rate as the pH was increased. This suggests that the rate of any test performed at pH 10 would have been quite slow, consistent with our data.

Examination of Figures 16 to 19 reveals no decrease in reaction rate with time, no suggestion of aggregates. Now that the influence of

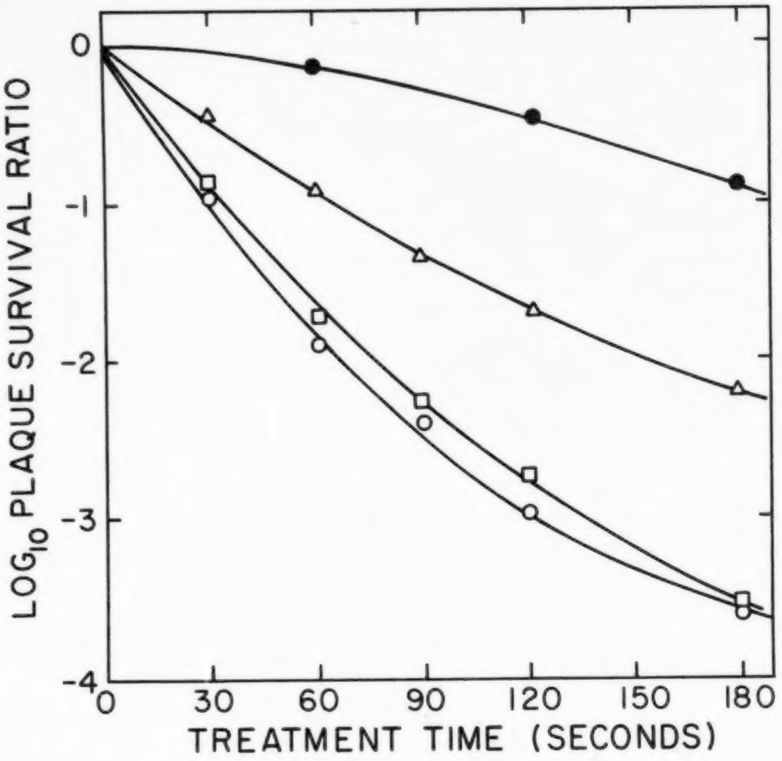

Figure 21. Inactivation of poliovirus at 20°C by OCl^- at pH 10, 3.5 μM (filled circles), 7.5 μM (triangles), 15 μM (squares) and 30 μM (circles.

aggregates has been removed, there is evidence of a short delay before inactivation reaches maximum velocity as though a definite time were required for reaction and penetration of the large number of HOCl molecules with and through the capsid of the virus, as in a diffusion-limited process. In addition to the rate differences between the inactivation of poliovirus by HOCl and OCl^-, there are qualitative differences which must be considered. The inactivation by HOCl at all temperatures tested was characterized by a short lag period, followed by a linear semilogarithmic inactivation rate. This was also seen with HOBr at 10 and 20°C. On the other hand, the inactivation of poliovirus by OCl^- at 14 and 30 μM was characterized by a decreasing rate of semilogarithmic curve, as was OBr^-. It appears that the reaction mechanisms may be substantially different between the two forms of each halogen, but similar when the two acid forms (HOCl and HOBr) are compared, or when the two ionic forms (OCl^- and OBr^-) are compared. While the data presented here will allow no conclusion on this possibility, the considerable rate and kinetic curve differences suggest it very strongly.

At OCl^- concentrations of 15 and 30 µM (1.07 and 2.14 mg/L), the reaction rate decreased with time below a survival level of 10^{-2}. This would be expected for a virus suspension containing aggregates of many sizes. However, evidence has been brought to define the level of aggregation that remained in the virus suspensions used in this work. Typical EM pictures such as Figure 2 show no more virions in groups than can be accounted for by accidental falling of one upon another during their deposition upon the film. Thus, aggregation is certainly not enough to influence the survival ratio above the level of 10^{-2}. Doubtless the best evidence that aggregates are not responsible for the decreasing reaction rate of OCl^- in the region of 10^{-3} survival ratio is that no such decrease was seen in this region with HOCl and the same virus.

Electron microscopy of negatively stained poliovirus particles (22) exposed to chlorine has shown considerable physical damage when applied at a concentration sufficient to cause loss of infectivity to a calculated level of 10^{-4}. At this level many particles appeared normal, that is, excluded PTA, although about one-half of the particles appeared damaged sufficiently to permit entry of the PTA. At the 10^{-6} survival level, some normal appearing particles were found. Since all tests performed for electron microscopy were allowed to incubate for 15 s, these results indicate that the loss of titer precedes the visual damage to the particles. The observed damage, therefore, is not the event which leads to inability to form plaques, but some event which is indistinguishable by electron microscopy is responsible for loss of titer. The effect of HOCl on poliovirus is different from that of HOBr on reovirus, as reovirus RNA was ejected from the particles, while the total number of particles remained constant. Reovirus capsids suffered some structural damage but they could still be recognized in the electron microscope.

It must be emphasized that the disinfection rates obtained here with carefully prepared suspensions of single virions are for purposes of comparison of one virus with another, one reagent with another, etc. Aggregates must be avoided in this work but aggregates will doubtless be present in practical sterilization of water in the field and so virus inactivation there must be slower.

SERIES III. THE COMPLEX KINETICS OF INACTIVATION OF ECHOVIRUS-1 WITH CHLORINE IN WATER (24)

Echovirus-1 (Farouk Strain) was produced in cultures of HEp-2 cells and stored unfrozen (4-6°C) in the sucrose solution in which it was purified. This sucrose solution was made with 0.01 M phosphate buffer containing 0.1 M NaCl. Stock virus was diluted with 0.14 M NaCl solution immediately before injection into the flowing stream of buffered HOCl solution for measurement of IR.

The inactivation of echovirus by 20 μM HOCl at pH6 and 20°C is shown as a function of time in Fig. 22. Several experiments were required to demonstrate clearly the two transition points of this complex curve. During the first second of exposure 98% of the PFU were inactivated. then no further change was observed until 15 sec, at which time somewhat less rapid inactivation was resumed, reaching the 10^{-4} level of survival in about 24 sec. Suspecting virion aggregation at this point, sodium chloride up to 0.3 M was added to the 0.01 M phosphate buffer in the chlorinated water and the experiments were repeated. The inactivation curve remained the same.

Virus was picked from plaques surviving 15 sec of HOCl exposure (plateau region of Fig. 22), and with this the inactivation experiment was repeated. These progeny of the surviving plaque gave the same response as the parent virus preparation. There was no initial lag, which would be expected from a more resistant mutant, and the time when inactivation was resumed was the same also.

For inactivation of echovirus by HOCl, pH 6.0 was chose in order that the results might not be complicated by the presence of chlorine in any other active form, Cl_2 or OCl^-. The virus proved to be isoelectric at pH 5.7 (Fig. 23), but there was no tendency for aggregation to occur in this region. Single particle approximation (SPA) tests carried out in several 0.05 M buffers covering the range of pH from 3 to 11 showed virion aggregation in the acid range below pH 5 but none above this point (Fig. 23). Electron microscopy also showed that the virus preparations at pH 6 (with and without added NaCl) contained very few aggregates. Virions attached to aluminum-coated collodion films by Brownian bombardment (KA method), revealed about 30 pairs per thousand, three triplets per thousand, and larger gropus in progressively smaller numbers. No clumps as large as 10 could be found. Furthermore, VAT tests of supensions of PFU that had survived 15 sec of HOCl treatment showed that they sedimented at the same rate as single virions. They were not aggregates, not even pairs.

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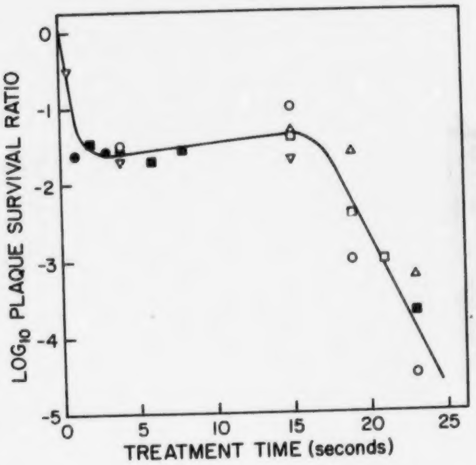

Figure 22. Inactivation of ehcovirus at 20°C µM HOCl at pH6. Different symbols indicate 6 repeat experiments.

Further evidence that virion aggregation does not influence the shape of the inactivation curve is supplied by repeating the experiment at a lower virion concentration. Most of the experiments were done with suspension of 10^8 virions/ml. When this was reduced to 10^7 virions/ml, the inactivation curve remained essentially the same. The persistent level was 99% and inactivation resumed between 15 and 19 sec.

When infectivity declines rapidly in a virus suspension containing a disinfecting agent, then the reaction slows or even stops, one looks for depletion of the active agent, or a fraction of the virus in large protective aggregates, or possibly a resistant fraction of the population that may be genetically different. Inasmuch as the reaction with echovirus begins again after a 14 sec delay at the 2% survival level, there is no question here of depletion of the HOCl.

It would take at least 16 spheres (virions) to surround one and so provide it with even one layer of protection. No aggregates this big were observed in any of the electron micrographs containing several thousand virions each. Furthermore the resistant PFU sedimented at the same rate as single virions. Still furthermore, virus progeny from resistant survivors have the same initial rapid reaction rate, the same plateau of resistance (survival level ca. 10^{-2}) and the same delay time until disinfection is resumed. This pattern was not altered when the virus concentration was reduced by a factor of 10. If aggregation were induced by destabilization of the virions suspension when it entered the HOCl solution, the rate of formation of aggregates would have to be proportional to the square of the

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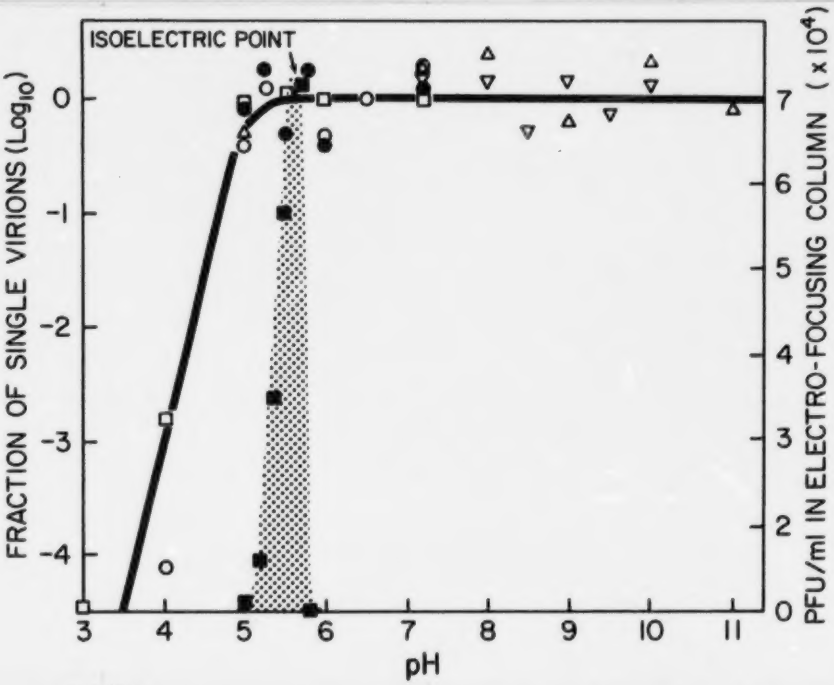

Figure 23. Isoelectric point of echovirus infectivity (filled squares) and rate of aggregation (several experiments, squares, circles and triangles).

virion concentration (25). Reducing this by a factor of 10 would have reduced the aggregation rate by a factor of 100.

These observations are sufficient to send us looking for a different survival mechanism of echovirus in HOCl. A few percent of the virion population is, or becomes, sufficiently resistant to sustain for 15 sec the concentration of HOCl that inactivated 98% of the population in 1 sec. If the resistant ones are just more difficult to penetrate, then the observed resumption of disinfection indicates that sufficient penetration of the HOCl has finally been achieved. Evidence has been presented by Mandel (26) and also by Fujioka and Ackermann (27) supporting the hypothesis that the poliovirus capsid proteins may exist in two or more different, possible metastable, conformational states. This evidence comes from observations of electrophoretic mobility in Mandel's experiments, in which he finds not one but two isoelectric points for poliovirus. We find only one pI for echovirus, but Fujioka and Ackermann find their evidence in the effects of cations on the reactivity of poliovirus with guanidine. They recognized four conformational states of the virion which were reversibly interchangeable and yet retained infectious potential.

We are therefore encouraged to propose that echovirus may exist in two different conformational states, one of which is substantially more resistant than the other to penetration by HOCl. But even the resistant state is ultimately penetrated under the conditions of these experiments.

Virions in the resistant state may be present initially or a fraction of the population may be converted to the more resistant conformation by the change in environment when they enter the flowing stream of chlorine water. Our experiments do not distinguish between these two alternatives, but they do suggest a new avenue of investigation of the complex problems involved in the inactivation of viruses in water by halogen compounds.

SERIES IV. PARTIAL REACTIVATION OF CHLORINE-TREATED ECHOVIRUS (28)

The resistance of viruses in water suspension to both physical and chemical inactivating agents has been shown to be greater if the virions are aggregated (2,5,29). Substantial enhancement of survival occurs even when only small clumps of virions (groups of 2 to 10) are present (3). It is difficult to imagine how such small groups of virions could provide any substantial protection to even one virus particle from a relatively infinite number of highly mobile chlorine molecules (HOCl) approaching from all directions. The experiment described here gives evidence in support of a different mechanism of virus survival due to aggregation which may operate predominantly when only small groups are involved. It was designed to determine whether aggregation induced after the exposure of echovirus in a highly dispersed state to HOCl in water had any effect on the plaquing efficiency of the virus. The results indicate that it does. There are many reports of revival or reactivation of ultraviolet-irradiated virus brought about through multiple infection of host cells, either by highly concentrated virus suspensions or by the presence of aggregates. This multiplicity reactivation (MR) has never been claimed to be a repair of damage to the virions, but rather a mechanism of mutal aid in the act of infection. Something of this kind appears to be taking place with chlorine-treated echovirus.

Purified echovirus at pH 7.2 was diluted four fold with distilled deionized water. Hypochlorous acid stock solution was diluted to a concentration that would reduce the plaque titer of the virus suspension by between 1 and 6 $\log_{10}$ units, and this was added to the virus mixture. After a 2-min treatment period, a non-chlorine-treated control and half of the chlorine-treated mixture were diluted fivefold with 0.05M phosphate buffer (KH_2PO_4-K_2HPO_4) at pH 7.2, and the remaining half was diluted with 0.05 M acetate buffer (CH_3 COONa-CH_3 COOH) at pH 4.5. Echovirus does not aggregate at pH 7.2, as shown by electron microscopy and differential centrifugation, but it will aggregate when the pH is lowered to below pH 5 (24). The mixtures were left standing for 4 h at room temperature to allow aggregation to take place. They were titrated for plaque formation on monolayer cultures of HEp-2 cells.

Three different preparations of purified echovirus, after velocity banding on a 10 to 30% sucrose gradient gave virion concentrations ranging from 6×10^{11} to 2×10^{12} per ml. Electron micrographs showed a state of dispersion indistinguishable from that of the poliovirus of Fig. 2. No clumps could be found with sufficient size to provide protection for one or more virions from contact with HOCl. An initial experiment was performed with one of the preparations at three pH values, 4.0, 4.5, and 5.0 in an effort to determine which produced the optimum degree of aggregation among the HOCl-treated virions to demonstrate reactivation if it was occurring.

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No substantial increase in survival titer was observed with samples adjusted to either pH 4 or pH 5 when compared with the chlorine-treated control at pH 7.2 (reduced 3 $\log_{10}$ units in titer). Adjustment of the chlorine-treated virus to pH 4.5 resulted in a 40 fold higher survival titer. No plaque enhancement of normal (untreated) virus was observed after the same treatment.

Following this evidence for superior plaque formation by the virus that had been adjusted to pH 4.5, many experiments were performed at a variety of survival levels ranging from 10^{-1} to 10^{-6}. Three experiments with one preparation (final particle count after adjustment to pH 4.5 was 2.0×10^{10} virions per ml) are shown in Fig. 24. A significant amount of revival occurred in each experiment, with greater initial $\log_{10}$ reductions resulting in proportionally greater revival in titer. The dotted curve shows the higher plaque survival based upon titration of the subsequently acid-treated virus.

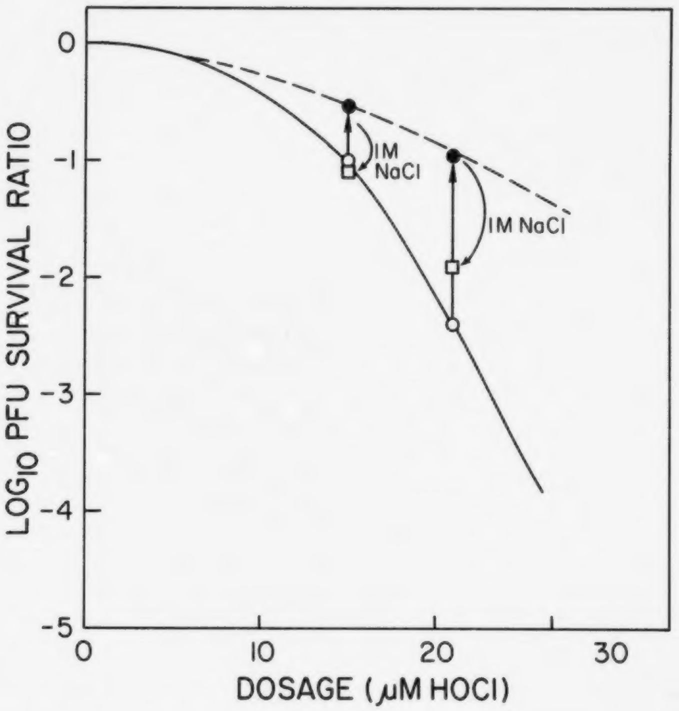

Figure 24. The degree of revival of echovirus infectivity becomes greater as the survival ratio decreases.

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The degree of enhancement of the survival titer after acid treatment (Fig. 25, ordinates) increased as the survival titer of the chlorine-treated control at pH 7.2 decreased (abscissa) for all three virus preparations studied. The different preparations showed different degrees of enhancement with maximal values obtained when the virion concentration during acid treatment was approximately 2 x 10^{10} particles per ml. Very dramatic increases occurred when the titer of the chlorine-treated control was reduced 5 to 1 $\log_{10}$ units. In one case, the titer rose 2.0 $\log_{10}$ units, or 800-fold, and in another the titer rose 3.4 $\log_{10}$ units, or 2,500 fold.

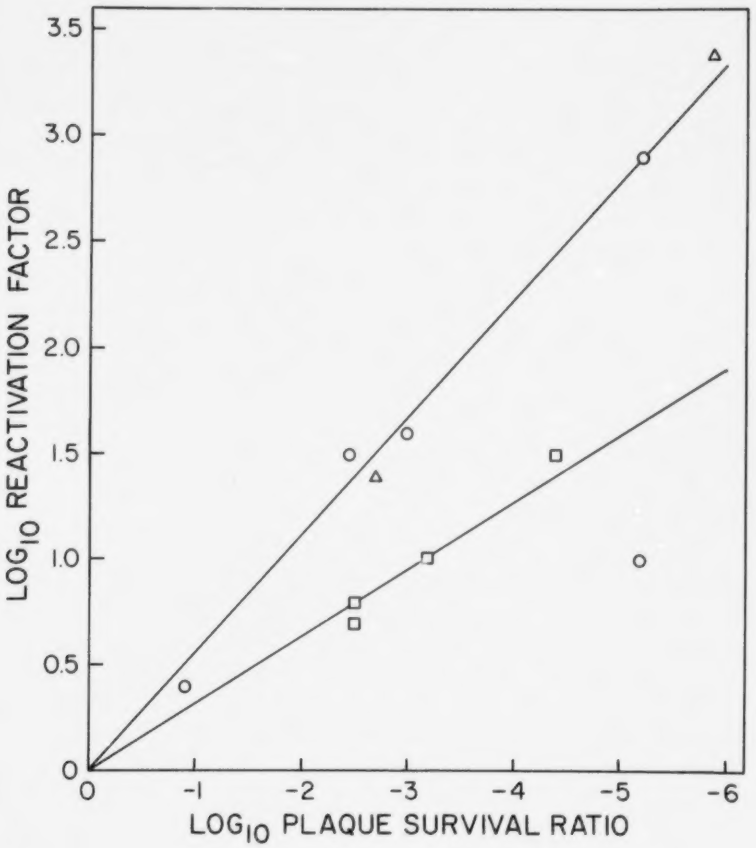

Figure 25. Relationship between PFU titer enhancement and plaque survival.

In its present state of development the experiment is somewhat difficult to manage, and one of the three preparations was to some extent unpredictable in the degree of revival produced, especially when the virus concentration was varied from the optimum value. The other two preparations gave very consistent and reproducible results. Possible explanations for this difficulty will be discussed in a subsequent section .

Although the data so far presented suggested that plaque enhancement depended on a critical amount of aggregation, the possibility remained that the acid buffer itself was affecting the virus particle, perhaps by changing its conformation (24,26), and was producing a more infecitous particle. This appeared unlikely, however, since at pH values very close to 4.5, no reactivation occurred. To further test this possibility, sodium chloride was added to the acid mixture to prevent aggregation from taking place, as it did with poliovirus at acid pH values (7). One molar sodium chloride eliminated most of the aggregation which would have been induced without it and it also reduced the degree of revival significantly (Fig. 26).

The particle concentration in a suspension affects the rate of aggregation of colloids according to the theory of von Smoluchowski (8,25). Virion concentration, as well as the time allowed for aggregation to take place, determines the resulting degree of aggregation. Figure 27 illustrates dramatically how the virus particle concentration can affect the degree of revival. When the particle concentration was optimal, 2 x 10^{10} particles per ml, a 2,500-fold increase in titer was observed. When the virus concentration was reduced to 10^{10} particles per ml, the plaque titer of the acid sample increased only 60-fold.

A series of experiments was performed at particle concentrations ranging from 4.5 x 10^6 to 4.5 x 10^{10}. Some increase in titer occurred in two of four experiments with a particle count greater than 3 x 10^{10}. Electron micrographs show that at this concentration, massive aggregation occurs under the conditions of these experiments, and any reactivation that occurs might be obscured by the decrease in the number of suspended units of virus. In 26 of 33 experiments in which the particle count ranged from 5 x 10^9 to 3 x 10^{10}, significant revival occurred, and at particle counts below 5 x 10^9, revival occurred in only 3 of 13 experiments.

Although electron microscopy gave qualitative data regarding the degree of aggregation at pH 4.5, it was not possible to use this method to determine the degree of aggregation that remained after the dilutions of the virus samples into phosphate buffered saline for titration, since such analysis requires a particle concentration of about 10^{10} virions per ml. Some dispersion of the aggregates seemed likely because of the higher pH and the presence of 0.14 M NaCl. To determine quantitatively the degree of aggregation remaining in the virus suspension when it actually reached the cell monolayers, virion aggregation tests were performed. These revealed that a significant residuum of small aggregates of 2 to 8 particles (blue section) had remained after readjustment to pH 7. It is mainly aggregates of this size that must have reached the cell monolayer and produced the apparent reactivation.

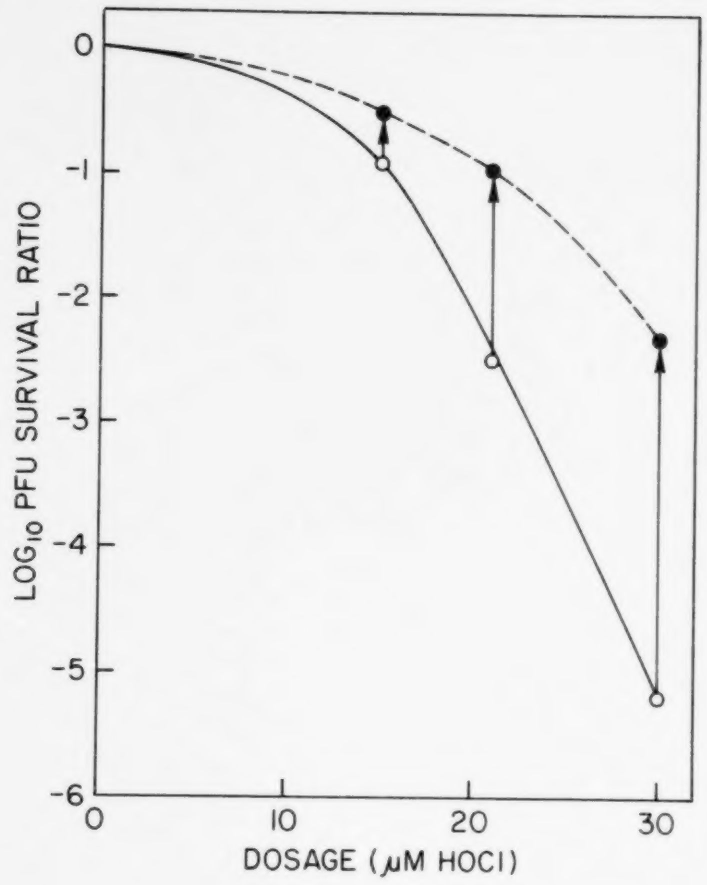

Figure 26. Revival of infectivity of echovirus is greatly reduced by addition of NaCl, which reduces virion aggregation.

Although it may be theoretically possible to achieve a reproducible degree of aggregation in a series of virus suspensions, it is very difficult to maintain this degree of aggregation during the steps required to bring the aggregates to the cell monolayer. As previously mentioned, both the presence of salt and neutral pH are conditions which favor dispersion of echovirus (24), and these are precisely the conditions required for plaque titration. The degree and rate of dispersion cannot be controlled, and this may account for the difficulties encountered in these experiments in consistently reproducing a certain degree of revival.

The electron microscope reveals some damage to the protein coat of the virion but this may have occurred after the inactivation. The fact that a substantial fraction of the virions retained their normal appearance after chlorine treatment suggests that the critical damage may be in the ribonucleic acid, although electron micrographs can only offer

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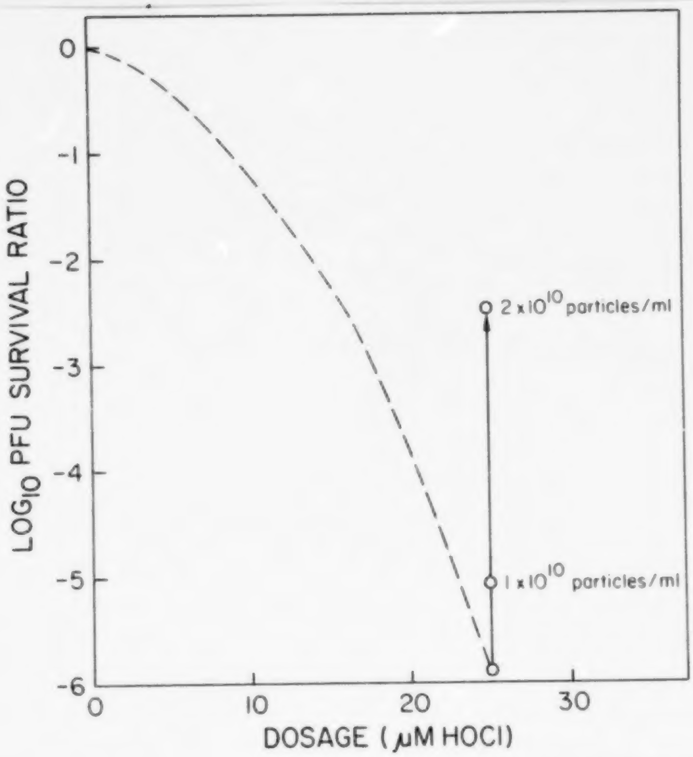

Figure 27. Effect of virion concentration on revival infectivity after chlorine treatment.

possibilities. It has been shown that inactivation of the poliovirus by ammonia involves a reaction with the ribonucleic acid (24), and that the ribonucleic acid is the site of inactivation of f2 virus by chlorine (W.H. Dennis, V.P. Olivieri, and C.W. Kruse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q55, p. 270). It is highly probable that the mechanism of inactivation of chlorine varies with the enteric virus under study, the pH, the ionic environment, and the chlorine species. Because so little is known about the mechanism of chlorine inactivation of echovirus, one can only speculate as to which of these interactions might be taking place in these experiments.

The results presented here suggest a topographical role in reactivation as previously mentioned in connection with the work of Abel (31). At the 10^{-5} survival level in these experiments, the multiplicity of particles was calculated to be 500 virions per cell. The probability that any two of these would reach the cell at the same spot of its surface is very small indeed. Five hundred particles would be approximately 70 diameters apart on the average. Furthermore, the virions arriving at the cell surface in a plaque titration do not arrive all at one time. It appears that there is something quite unique about plaque titration of aggregated virions; it reveals of an otherwise rare coincidence involving both place and time.

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The possibility exists that no synergistic effect is taking place, but, rather, the aggregates, by virtue of their size or some other attribute, are more efficient plaque producers. Whatever causes only 1 in 200 enteric virus particles to be infectious may be overcome in some way by aggregation. In the course of this and related work involving plaque titration of thousands of preparations of poliovirus and echovirus, no enhancement of the titer of fresh virus (MA has ever been associated with aggregation. Such an effect was observed (32) with vaccinia virus in this laboratory several years ago, but no evidence of multiplicity activation has occurred with either of the above-mentioned enteric viruses. It would appear that aggregates of fresh (undamaged) echo virions are not significantly better plaque producers than single virions.

A mechanism of repair by exposure to visible light (33) and a phenomenon called host-cell reactivation have been demonstrated (34) whereby ultraviolet-irradiated, DNA-containing virus particles can regain a substantial portion of their lost infectivity, but neither of these repair mechanisms has been observed with the chlorine reaction on the picornaviruses. If any such mechanism is occurring in these experiments, reactivation due to the formatin of the aggregates occurs over and above this effect, since any such repair mechanism would be expected to occur equally well with the dispersed chlorine-treated control.

The possibility that the protein coats of poliovirus and other enteric viruses can exist in two or more different infectious conformational states has been suggested by several investigators (26,27,35), and specifically it has been suggested for this type and strain of echovirus (24). Therefore, the possibility that the acid itself was stabilizing the particle in a more infectious conformational state was considered. The fact that reactivation did not occur at other pH values close to 4.5, together with the observations that the addition of salt at pH 4.5 prevented enhancement from occurring and that revival was critically dependent on the virus particle concentration, led us to conclude that a conformational alteration was not the cause of the titer enhancement.

These experiments involve quite high virus concentrations, and the concentration of viruses in water arriving at water treatment plants is not nearly so high. Aggregation would not be expected to occur among single particles in so dilute a system. However, the natural state of some enteric viruses in water appears to be aggregated. Freeze-thaw release of aggregates of poliovirus (5) and also echovirus produces a suspension of single particles and large and small aggregates, as well as some association with cell debris. Dilution of natural waters results in little, if any, breakup of these aggregates (5). It is highly probable that many of the enteric viruses reach the water treatment plant in an aggregated state. To demonstrate reactivation in the laboratory, it is necessary to begin with a highly dispersed suspension of virus, to eliminate any possibility of protection, and then subsequently induce aggregation. This requires a high concentration of virus particles. Lower concentrations in the field would not preclude reactivation from occurring as long as the virus was already in the aggregated state. It would just make the virus appear to be more resistant to chlorine treatment.

The effect we have found is difficult to demonstrate because the only way we have found to produce quick, if not quantitatively predictable, aggregates in a small volume is by making the medium slightly acid. In the region of pH 4.5 the rate of coagulation of echovirus is a very critical function of pH. A slight shift in pH (or virus particle concentration) can make a great change in the quantity and size of the aggregates formed in the 4-h aggregation period and also in the number and size that remain after the final pH and salt adjustment that occur in the dilution series for plaque titration. Despite the difficulty involved in the demonstration, the fact that small aggregates are more efficient plaque formers than single damaged particles suggest that reduction in the number of suspended units of chlorine damaged virus can result in an increase in infectivity. Virus exposed in the aggregated state to a disinfecting agent may exhibit superior resistance through this effect, in addition to influences such as protection (29).

If this mechanism of virus survival is occurring in natural waters, it has important implications beyond increasing the resistance of virus to chlorination. Current virus concentration procedures involve virus elution at alkaline pH and dilution in physiological buffers for titration, both of which tend to disperse the enteric viruses under study in this laboratory. Infectious aggregates from treated water may be dispersed during the concentration procedure, and yield low or negligible evidence of infectivity.

In addition, multiplicity reactivation has been demonstrated not only in tissue culture, but also in the cells of an organized host (36), suggesting that the superior plaquing efficiency of chlorine-treated echovirus aggregates in tissue culture might parallel a similar increased efficiency in humans.

SERIES V. VIRION AGGREGATION AND COLLOIDAL STABILITY

Quantitation and Kinetics of the Aggregation of Poliovirus and Reovirus (8)

Scattered observations had been made earlier in this work indicating the influence of such variables as ionic strength, type of salt and pH on the physical stability of virus suspensions. Ultimately it became imperative that a careful study of these effects be an essential part of this project. The SPA test as first devised (7) had defects that were revised (8) and the form described here in materials and methods was used in all of the following experiments.

Typical kinetics of aggregation data are shown for poliovirus (Fig. 28A) and for reovirus (Fig. 28). The similarity of these experimental curves with those predicted for colloidal particles in general by von Smoluchowski (25) is readily seen. The general formula that he derived to express the maximum rate (100% adhesion of all colliding particles) of coagulation is as follows:

$$N_k = \frac{N_o \left(\frac{t}{t_{1/4}}\right)^{K-1}}{\left(1 + \frac{t}{t_{1/4}}\right)^{K+1}} \qquad (1)$$

where N_k is the number of aggregates of size k, N_0 is the initial number of particles (assumed to be all single particles), t is the time in seconds, and $t_{1/4}$ is the time for aggregation to proceed to a point at which exactly one quarter of the single particles remain. (Von Smoluchowski used "T" to refer to this time point (25), which is also the same as the time for the total number of aggregates plus single particles to reach one half of the initial number, N_0, We used "$t_{1/4}$" here in reference to single particles only and to prevent confusion with T= temperature.). The value $t_{1/4}$ is calculated according to the following equation:

$$t_{1/4} = \frac{3\eta}{4N.kT} \qquad (2)$$

where η is the absolute viscosity of the medium in pois, k is Boltmann's constant (1.38×10^{-16} erg/degree), and T is the absolute temperature. When k = 1, that is, single particles, $N_k = N_1$ equation (1) is reduced to the following:

$$N_1 = N_o \frac{1}{(1 + t_{1/4})^2} \qquad (3)$$

and the number of single particles remaining at any time t can be calculated.

The efficiency of aggregation can be calculated by the use of the factor ε according to the equation:

$$\varepsilon = \frac{\text{Calculated titer}}{\text{Observed titer}} \qquad (4)$$

and can be related to the aggregation equations above by insertion into equation 3 (as demonstrated by Hartman [37]) as follows:

$$N_{1(obs)} = N_o \frac{1}{\left(1 + \frac{\varepsilon t}{t_{1/4}}\right)^2} \qquad (5)$$

With respect to the factor ε, we suggest two definitions for its use: (i) the efficiency factor ε^2, which gives that portion of collisions which are effective in producing a permanent aggregate; and (ii) the time reduction factor ε, which, when applied as in equation 5, alters the result to closely approximate the amount of aggregation actually observed. Under the conditions employed in this experiment ($\eta = 0.009$ poise; $\tau = 298^oK$) the average efficiency was 25% ($\varepsilon^2 = 0.2587$) indicating that one out of four collisions of single particles was effective in producing a permanent aggregate.

Quantitative aggregation of reovirus. Figure 28B shows the results of a similar test with reovirus aggregated at pH 4.0 in 0.05 M acetate buffer. Again, the aggregation was generally parallel to the calculated values, but not as rapid. The overall efficiency was close to 50% indicating that one out of every two collisions was effective in producing an aggregate. The points defining the observed data curve in Fig. 28B are much more widely spread than those in Fig. 28A. This is characteristic of aggregation experiments and results from two main causes: (i) slight differences in efficiencies within each tube and (ii) variation among tubes resulting from

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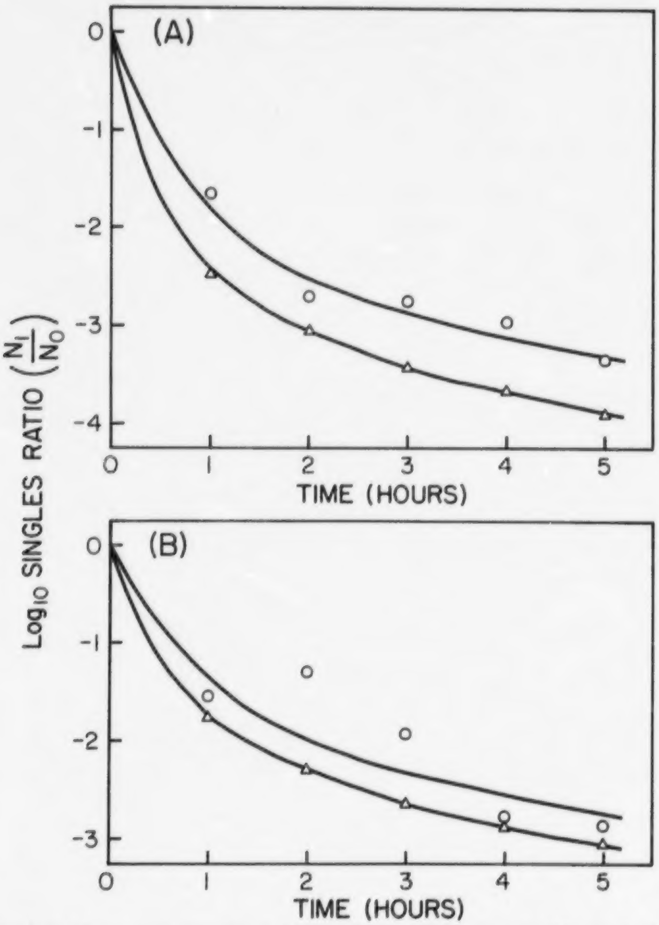

Figure 28. Rate of aggregation (A) poliovirus at pH3 and (B) reovirus at pH4. Values calculated for maximum (25), triangles.

mechanical factors in the handling of the tubes, buckets, rotor, pipettes, etc. The second factor probably contributes more variation within an experiment due to the lack of any stabilizing material, such as a density gradient, in the virus suspension.

Table 3 shows several values for the efficiency ($\varepsilon 2$) of aggregation when each of the two viruses were placed in various buffers at different pH values. The $\varepsilon 2$ values were calculated according to equation 4 at the times given in the table. The total amount of aggregation which took place under each condition was dependent on time, temperature, viscosity, and virus particle concentration, but the efficiencies which are calculated for each set of conditions can be seen to vary from a low of 0.38 to almost 90%. In the case of poliovirus in borate buffer at pH 9, the time value taken was only 1.5 h because aggregation was so rapid as to preclude taking samples

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at much longer times. However, in this particular case, repeat experiments at similar or slightly lower particle concentrations in borate buffer at pH 9.0 confirmed the efficiency of > 90% at longer time values.

TABLE 3. Efficiencies of aggregation (ε^2) for poliovirus and reovirus and in buffers at various pH values

Virus	Buffer (0.05M) and pH	Time (h)	Efficiency of aggregation (ε^2) (%)
Poliovirus	Borate, pH 9.0	1.5	87.9
	Glycine-hydrochloride pH 3.0	4.0	19.9
	Acetate, pH 5.0	4.0	3.03
	Phosphate, pH 6.0	4.0	0.4
Reovirus	Acetate, pH 4.0	4.0	63
	Glycine-hydrochloride pH 3.0	4.0	2.5
	Acetate pH 5.0	4.0	0.38

EFFECTS OF SALTS ON AGGREGATION OF POLIOVIRUS AND REIOVIRUS AT LOW pH (38)

The basic underlying mechanism which governs the aggregation of virus particles and their adsorption to other particulate matter involves the nature of (i) the soluble ionic groups with the virus in suspension (such as Na^+, Cl^-, etc.), (ii) the charged groups on the surface of the virus particle (the isoelectric point of the virus is the single most important overall reflection of these groups), and (iii) the existing ionic double layer (39,40) which is the result of the interaction of the first two. The ionic double layer is quite marked by affected the pH, ionic composition of the medium, and the isoelectric point of the virus. Therefore, an examination of the effects of ionic species such as Na^+, Mg^{2+}, Cl^-, Al^{3+}, as well others, on the aggregation of virus particles induced by low pH should provide some understanding of the nature of virus aggregation and adsorption.

To examine the effects of salts on the aggregation of viruses, it was essential to know the isoelectric point of each virus. Accordingly, the isoelectric points were determined in an LKB isoelectrophoretic apparatus as described above, and the results are shown in Fig. 29 and 30. In a pH gradient of 3.5 to 10, Mahoney poliovirus infectivity (Fig. 29A) was focused at a pH of 8.2 ± 0.1. There was also a small shoulder at the base of the main peak at a pH of 7.7, and it was thought that this shoulder might represent a second peak of infectivity similar to that reported by

Mandel (26) for poliovirus. However, when the same preparation of virus was subjected to electrophoresis on a pH gradient of 7 to 9 (Fig. 29B) only one main peak of pH 8.3 ± 0.1 was found, indicating the artificial nature of the lower shoulder found in the pH gradient of 3.5 to 10. This isoelectric point for the Mahoney strain is considerably different from the A forms at or near pH 7 as reported by Mandel (26), although it is similar to the A form of a large (L) plaque varient of Mengo virus (41).

On a gradient of pH 3.5 to 10, the isoelectric point of reovirus III, Dearing strain, was found to be pH 3.9 ± 0.1, and no other peaks of infectivity were found (Fig. 30).

The examination of the effects of salts on the aggregation of poliovirus and reovirus was performed in conjunction with the isolelectric focusing data. Because the isoelectric point of reovirus was at pH 3.9, these particles would possess a net positive charge at pH values below this level, and a net negative charge at pH values above it. Furthermore, in buffers at pH 3 and 5, both reovirus and poliovirus aggregate markedly (7). It was possible, therefore, to study the effects of salts on the surface of the reovirion under two different overall charge configurations and to compare these effects with those on poliovirus, which is very strongly positively charged at both of these low pH values. For these reasons, the pH values of 5 and 3 were chosen for the work on salts and aggregation of both viruses. Subsequent work on the aggregation of poliovirus and reovirus at high pH values greater than 7 will be reported later.

The examination of effects of salts on aggregation of each virus utilized the following SPA test: six cellulose nitrate tubes (0.5) by 2 inches [1.27 by 5.08 cm]) were filled with 5 ml of the buffer under study, five of the tubes containing increasing amounts of salt and the sixth tube with low pH buffer only. At zero time the virus was added in microliter amounts to a final concentration of 0.5 X 10^9 to 1.0 X 10^9 particles per ml, and all tubes were covered and incubated at room temperature ≈24°C for 4 h. All tubes were then placed in the SW50.1 rotor and cnetrifuged as previously described (7). The top 2.3 ml was carefully removed and neutralization served two purposes; (i) because it was usually necessary to plate out the undiluted material to obtain countable plaques, a pH at or near 7.2 was essential, and (ii) the addition of the glycine at pH 11.7 allowed for a check by back-titration of the pH of those tubes containing large concentrations of salts. In all cases, the pH in the salt-containing tube was identical to that in the pH control tube.

After the neutralization, the suspensions were plaque titrated, and the final titers were recorded in $\log_{10}$ relation to the control tube without salts at low pH, used as the zero reference values. with this method of plotting the data, positive values represent an increase in single particles and, hence, a decrease in aggregation; conversely, negative values represent a decrease in single particles in the top 2.3 ml after centrifugation. In the absence of salt, the aggregation of both viruses at pH 5 was -2.0 to -2.5 $\log_{10}$, whereas that in pH 3 buffer was -2.5 to -3.0 $\log_{10}$. This data has not been entered on the figures because it varied somewhat from test to test, but it does indicate the average

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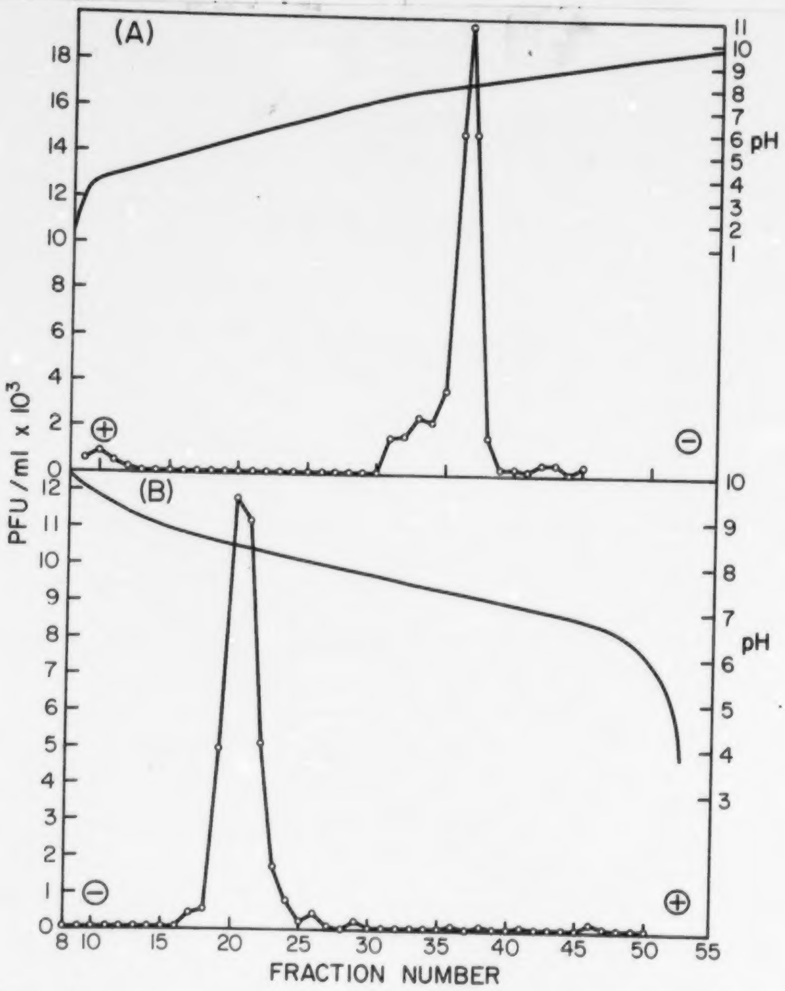

Figure 29. Isoelectric focusing of PFU of poliovirus (Mahoney) (A) pH region 3.5 to 10 and (B) pH region 7 to 9.

amount of aggregation obtained at low pH alone.

Figure 31 shows the effects of $MgCl_2$ on the aggregation of poliovirus and reovirus at pH 5 in acetate buffer and pH 3 in glycine buffer. At pH 5 (Fig. 31A) there was an initial enhancement of aggregation of poliovirus at a low concentration of 0.002 M $MgCl_2$, but as the salt concentration was increased, aggregation was markedly inhibited to values as high as 1.0 M $MgCl_2$. Reovirus, on the other hand, showed no initial enhancement even at $MgCl_2$ concentrations of 0.005 M, but as the concentration of $MgCl_2$ was increased, aggregation was gradually inhibited. Although a smooth curve

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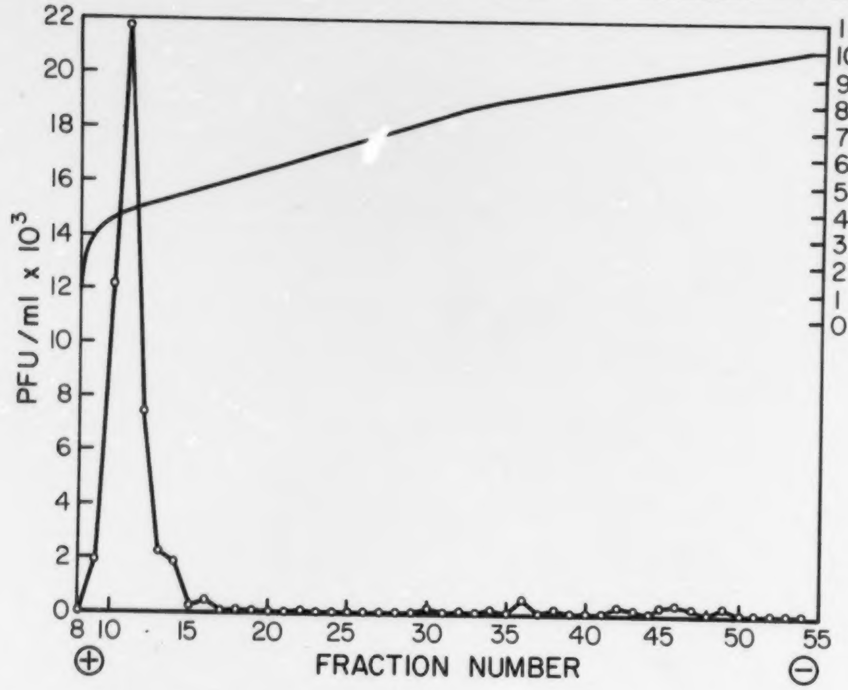

Figure 30. Isoelectric focusing of PFU of reovirus III (Dearing).

has been drawn through the points to approximate the best fit, it is important to note the rather wide distribution of the points used as the basis for drawing a curve. This variance results from a slight difference in the efficiency of aggregation from experiment to experiment. The net result was that the significance of each of the curves presented in this manner lies in the general shape of the curve, not in the absolute values of each of the points.

In Figure 31B, the aggregation of poliovirus at pH 3 in $MgCl_2$ has been taken from electron microscope data. This method is less quantitative, but it is possible to demonstrate that < 1% of the particles are in aggregates, hence this has been plotted as +2.0 $\log_{10}$. Reovirus showed the same inhibition at low $MgCl_2$ concentrations as did poliovirus, but it reaggregated when the $MgCl_2$ concentration was raised above 0.4M. No further inhibition was found at higher concentrations of $MgCl_2$. All reovirus data was generated by the SPA test.

The effects of $CaCl_2$ on virus aggregation at low pH are shown in Fig. 32. The effects on poliovirus (Fig. 32A and B) were similar to $MgCl_2$, except that the initial enhancement of aggregation at pH5 in 0.02 M $CaCl_2$ was not as marked as with $MgCl_2$. Reovirus at pH 5 (Fig. 32A) showed an enhancement of aggregation in the presence of 0.02 to 0.04 M $CaCl_2$ not shown with $MgCl_2$, but at higher concentrations aggregation was inhibited markedly. At pH3, (Figure 32B) reovirus showed the same peak of inhibition at 0.2 to 0.25M $CaCl_2$ as with $MgCl_2$ and rapidly reaggregated at higher

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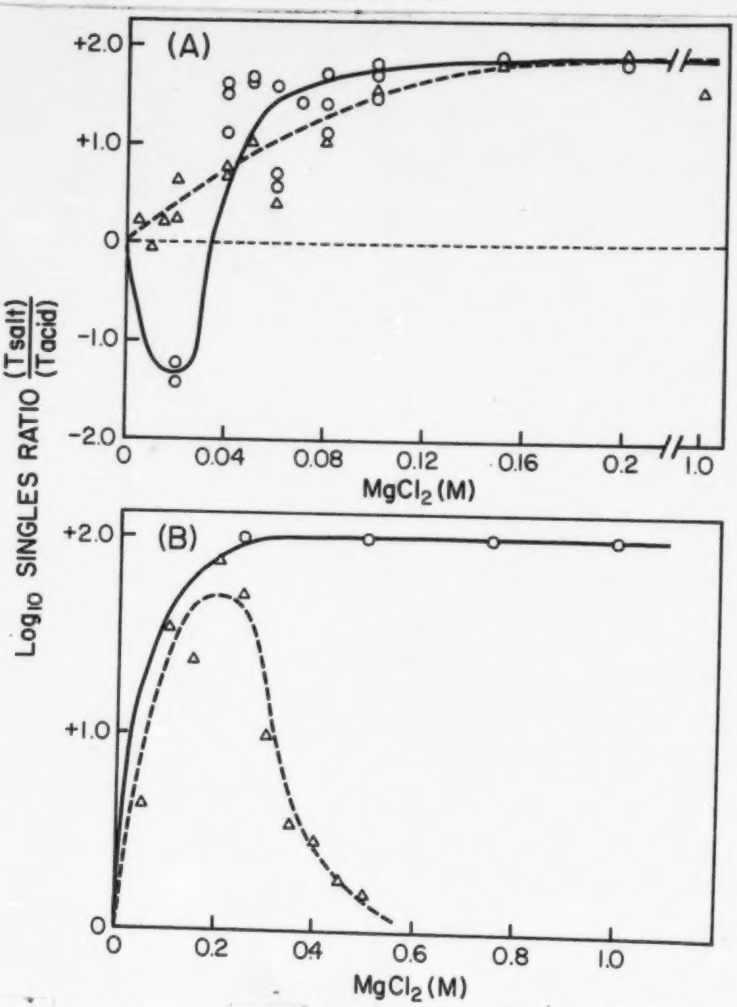

Figure 31. Aggregation of poliovirus (circles) and reovirus (triangles), (A) at pH 5 and (B) at pH3 in the presence of $MgCl_2$.

$CaCl_2$ concentrations.

Figure 33 shows the effects of $AlCl_3$ on the aggregation of poliovirus and reovirus. Aggregation in the presence of the trivalent cation Al^{3+} suffers even more from the wide variatin in values between experiments than those with divalent cations, so it has been necessary in some cases to use average values rather than individual experiments in the plotting of $AlCl_3$ data; this is especially true at pH3. Generally, aggregation in the presence of $AlCl_3$ was greater than in the absence of the salt, although reovirus aggregation seemed to be slightly inhibited at pH 3. Poliovirus consistently showed a marked increase in aggregation at very low levels of $AlCl_3$, between 0.001 to 0.01 mM; thereafter, aggregation was generally about -0.35 to -0.75 $\log_{10}$. Reovirus aggregation was extreme at pH 5 in the higher concentrations tested (>0.05 mM): this was probably due to

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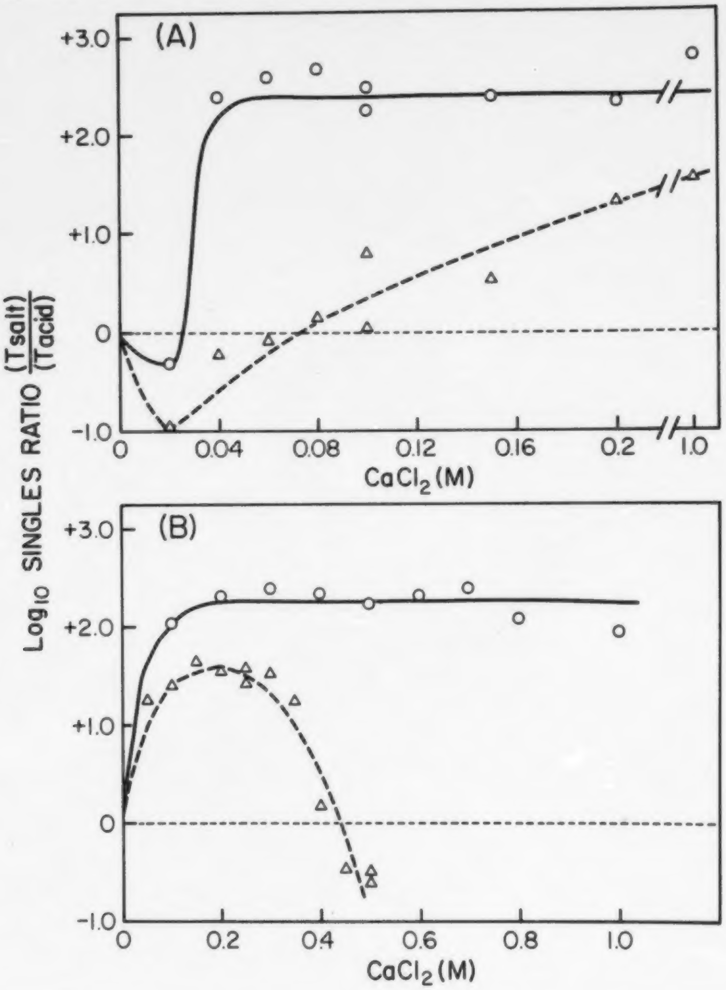

Figure 32. Aggregation of poliovirus (circles) and reovirus (triangles), (A) at pH5 and (B) at pH3 in the presence of $CaCl_2$.

attachment of particles to the insoluble $(AlOH)x^{2+}$ flock formed at this pH. Such a flock is very much reduced at pH 3, where Al^{3+} ions are considerable more soluble, and the effects on the reovirion produced an inhibition of aggregation.

The effects of specific salts as demonstrated in the previous section strongly suggested that the positively charged cation played a more important role in disruption or enhancement of aggregation than did the negatively charged anion. This was suggested primarily by the much greater effects of the aluminum ion Al^{3+} than the divalent Mg^{2+} or Ca^{2+}. Furthermore, monovalent ions as Na^+ in the form of NaCl required concentrations greater than 1 M to prevent aggregation at pH 3 with both poliovirus and reovirus, but lower concentrations of 0.2 to 0.6 M to

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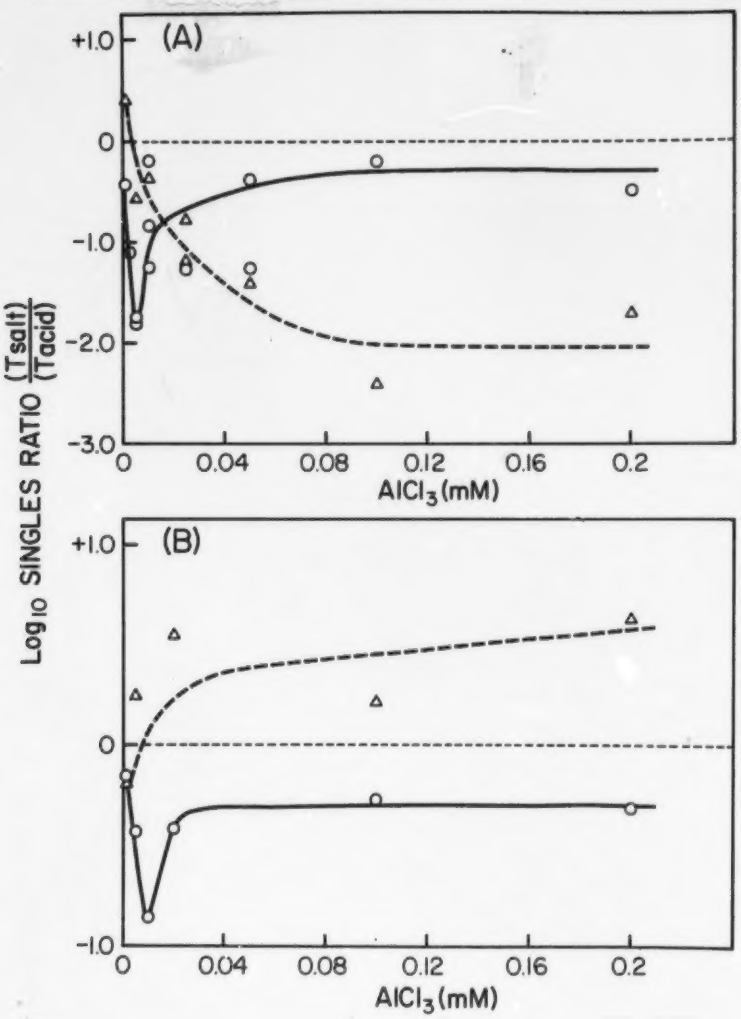

Figure 33. Aggregation of poliovirus (circles), and reovirus (triangles), (A) at pH5 and (B) at pH3 in the presence of $AlCl_3$.

prevent aggregation of these viruses at pH 5 (7). To examine this effect further, several experiments were performed on reovirus at pH 5 and 3 and poliovirus at pH 3 with monovalent and divalent cations and anions. The results of this study are shown in Figs. 34 and 35. At pH 5 (Fig. 34A), the reovirion carried a net negative charge, and inhibition of aggregation was obtained by significantly lower concentrations of the divalent Mg^{2+} ion than the monovalent Na^+ ion, regardless of whether the cations were in the sulfate or chloride form. At pH 3 reovirus carried a net positive charge, but Fig. 34B shows that at this pH inhibition of aggregation was again more sensitive to the divalent Mg^{2+} than to the divalent SO_4^{2-}. Magnesium sulfate produced the typical paraboloid curve of inhibition-aggregation at

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pH 3 (compared with $MgCl_2$ in Fig. 31B and $CaCl_2$ in Fig. 32B), whereas Na_2SO_4 at the same concentrations caused a slight but measurable increase in aggregation, about 0.3 $\log_{10}$, and had no effect on disruption of aggregates even when carried out to 0.5 M. Sodium chloride produced a slight increase in single particles at pH 3, but the effects fell off after 0.5 M, and no further effect was noted when the NaCl concentration was increased to 1.0 M.

Figure 35 shows similar results with poliovirus at pH 3. At this pH, poliovirus has a strong positive charge, yet the Mg^{2+} cation, either in the form of $MgSO_4$ (Fig. 35) or $MgCl_2$ (Fig. 31) was more effective in inhibiting aggregation than was the SO_4^{2-} anion, in the form of Na_2SO_4.

BUFFER EFFECTS IN THE AGGREGATION OF POLIOVIRUS AND REVOVIRUS AT LOW AND HIGH pH (42).

In some earlier experiments we used the following buffers at pH values of ≤ 7 to induce aggregation of virus particles: at pH 7 and 6, 0.05 M phosphate; at pH 5 and 4, 0.05 M acetate; and at pH 3, 0.05 M glycine hydrochloride. The amount of aggregation produced was generally proportional to the hydrogen ion concentration (that is, the lower the pH, the greater the amount of aggregation). To quantitate the aggregation produced by each buffer on both poliovirus and reovirus, 4 hour SPA experiments were performed on each virus in each of the buffers listed above. Briefly, this SPA test was conducted as follows. The virus was added to 5 ml of each buffer and to a control of PBS in cellulose nitrate centrifuge tubes (0.5 by 2 inches [1.27 by 4.08 cm]) to a final particle count of 10^9 particles per ml and allowed to remain at room temperature for 4 h. The tubes were then centrifuged at 30,000 rpm (poliovirus) or 15,000 rpm (reovirus) at 20°C for 20 min in a Beckman SW50.1 rotor in a Sorvall OTD-2 ultracentrifuge, using the "Reograd" mode at the termination of the run. The top 2.3 ml from each tube was titrated, removed, throughly mixed and the final titers were converted to $\log_{10}$ form and related to the control tube of PBS as the following ratio: $\log_{10}$ titer at pH <7 minus $\log_{10}$ titer in PBS (Fig. 36). Relative to the PBS control, the aggregation of the two viruses presented some similarities and some important differences. Poliovirus was quite sensitive to the effects of phosphate buffer at pH 6, whereas reovirus was not (7,38). Both viruses showed negligible aggregation at pH 7 in phosphate, however. Acetate buffers, whether at pH 5 or 4, caused marked aggregation than pH 4, in the case of poliovirus aggregation in acetate buffer at pH 4 was greater at pH 3 in glycine.

The variation in aggregation, which appeared to be dependent to a great extent on buffer type as well as pH, was examined in further detail in a series of SPA experiments with both viruses in several buffers at pH 6, 5, 4, and 3. The SPA tests were performed as described above, and the results are shown in Table 4 as the amount of aggregation relative to the control in either PBS or Tris-buffered saline. (Preliminary tests indicated that both PBS and Tris-buffered saline hold either virus in essentially monodispersed state; thus, either is suitable as a reference

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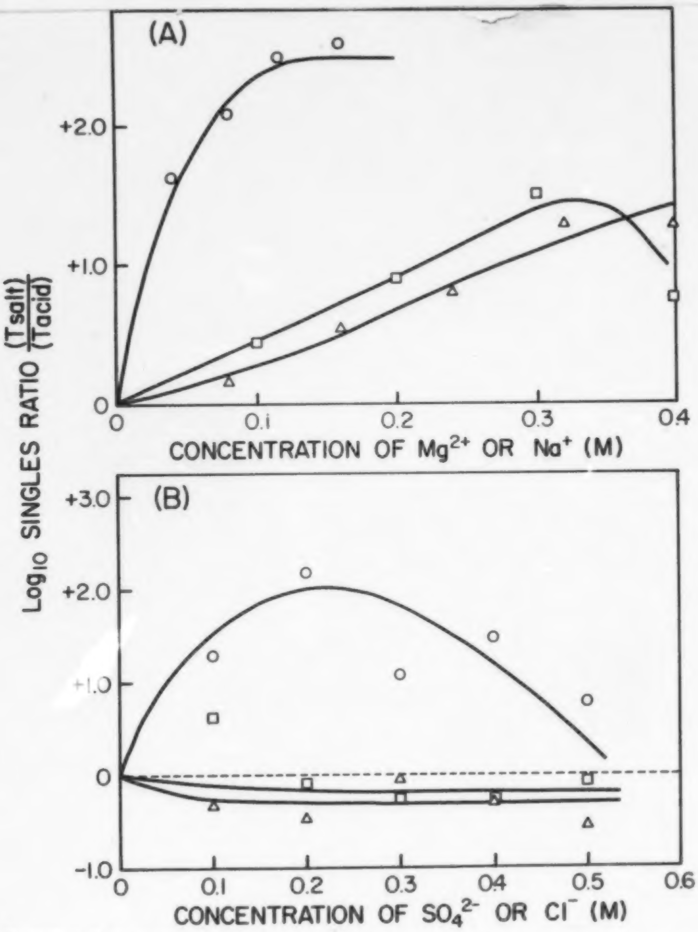

Figure 34. Effect of monovalent and divalent cations on the aggregation of reovirus (A) at pH5 and (B) pH3, circles $MgSO_4$, triangles Na_2SO4 and squares, NaCl.

control in SPA tests.)

The amount of aggregation at any given pH was clearly dependent on the buffer used. With poliovirus, citrate buffer at pH 6 (prepared by adjusting 0.05 M citric acid to pH 6 with NaOH) and McIlvaine phosphate-citrate (a mixture of $0.2MNa_2HPO_4$ and 0.1 M citric acid (43) induced the least aggregation (-0.42 and -0.48 $\log_{10}$, respectively. Acetate buffer, on the other hand, induced -2.64 $\log_{10}$ of aggregation, which was the greatest amount of aggregation induced by any buffer at pH 6. Other buffers were intermediate between citrate and acetate. Acetate buffer, in fact, was a strong inducer of poliovirus aggregation at all pH levels where it is tested. Phosphate buffer at pH 6 containing 2 mM ethylenediaminetetraacetate induced aggregation similar to that produced in phosphate alone, indicating that the chelating activity of such buffers as citrate and succinate probably was not a factor in the induction of aggregation.

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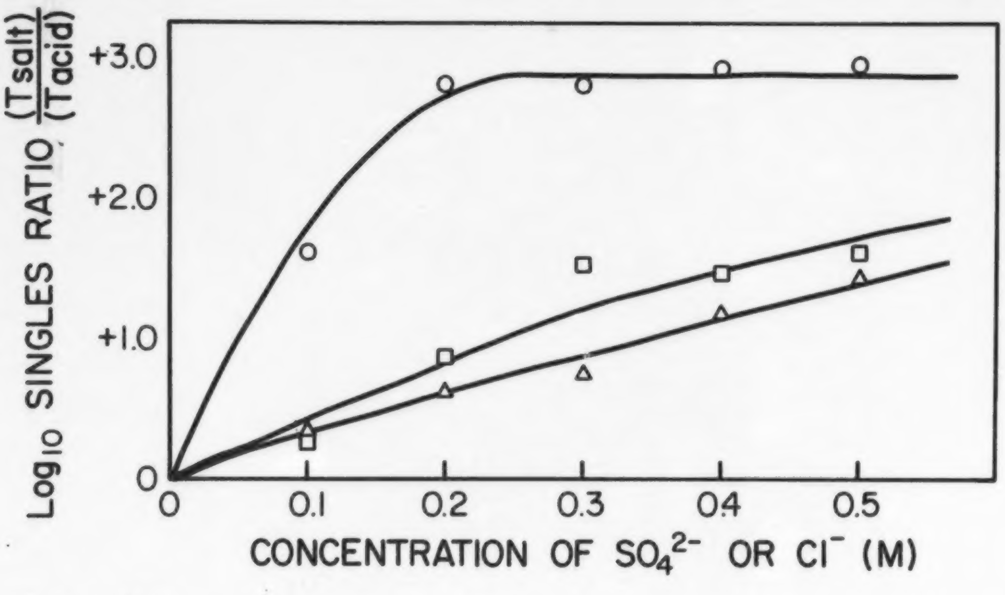

Figure 35. Effect of monovalent and divalent cations on the aggregation of poliovirus at pH3, circles $MgSO_4$ and squares NaCl.

The average amount of aggregation at each pH value increased as successively lower pH levels were tested. This increase may be a reflection of the activity of the anion of the buffer in the compression of the ionic double layer of the virus particles leading to aggregation.

Reovirus aggregation at low pH presented results somewhat different from those of poliovirus. At pH 6, reovirus aggregation was minimal, especially with phosphate buffer, with or without ethylenediaminetetraacetate, consistent with earlier results (7). Citrate buffer was also a weak inducer of reovirus aggregation, whereas acetate yielded the greates (-0.51 $\log_{10}$) amount of aggregation. Acetate, however, was not consistently among the greatest inducers of reovirus aggregation, although it was generally above the average value. At the lower pH values (pH 4 and 3) buffers such as succinate and citrate, as well as Tris-acetate were strong inducers of aggregation. Phosphate-citrate was generally weak at all levels tested.

In contrast to poliovirus, the average values of reovirus aggregation reached a minimum -2.9 $\log_{10}$ at pH 4 and was not increased at pH 3. This may be related to the fact that the isoelectric point of this strain of reovirus is at pH 3.9, and suggests that reovirus aggregates maximally at or near its isoelectric point, a fact not recognized until these quantitative data were obtained.

The preceding results strongly suggest an effect on the virus of the buffer system at any given pH value, but most buffers are limited in pH

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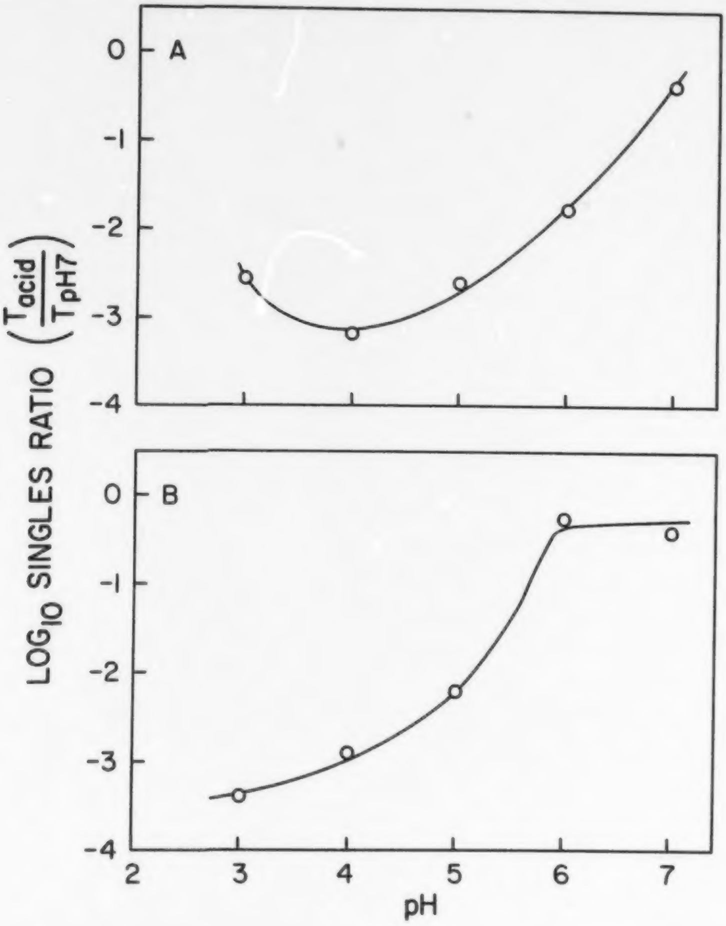

Figure 36. Aggregation of (A) poliovirus and (B) reovirus in the acid pH range.

range and can buffer effectively only through 2 to 3 pH units. To gain a better understanding of the relationship between the aggregation at any pH value and that at another value, and between different types of buffers, several SPA experiments were performed with phosphate-citrate and Tris-citrate buffers and both poliovirus and reovirus. These two wide-range buffers were used since they were capable of producing buffered pH values from 7 to 3. The results of these experiments are shown in Figs. 37 and 38. Figure 37 shows the aggregation of poliovirus and reovirus in phosphate-citrate buffer. Both curves were sigmoid in character due to the much greater aggregation at pH 4 and 3 than at pH 7 to 5. Poliovirus aggregation at pH 6 was negligible compared with that in phosphate alone (compared with Fig. 36A and Table 4), as was aggregation at pH 5 compared with that in acetate. The curve for reovirus (Fig. 37B) was similar to that in Fig. 36B with respect ot pH 6, but aggregation in phosphate-citrate at pH 5 was much less than in acetate at pH 5.

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TABLE 4. Aggregation of poliovirus and reovirus in various buffers at pH 6, 5, 4, and 3, as determined by a 4-h SPA test[a]

PH	Buffer type[b]	Poliovirus aggregation[c]	Reovirus aggregation[c]
6	Phosphate	-1.73	-0.13
	Acetate	-2.64	-0.51
	Citrate	-0.42	-0.14
	Succinate	-1.86	-0.20
	Phosphate + 2mM EDTA[d]	-1.64	-0.09
	Phosphate-citrate	-0.48	-0.18
	Tris-citrate	-1.48	-0.42
	Average at pH 6	-1.46	-0.24
5	Acetate	-3.65	-2.74
	Citrate	-2.50	-0.37
	Succinate	-3.59	-0.89
	Phosphate-citrate	-1.36	-0.47
	Tris-citrate	-1.52	-1.15
	Average at pH 5	-2.52	-1.12
4	Acetate	-4.47	-2.53
	Glycine	-1.27	-1.79
	Citrate	-2.40	-3.16
	Succinate	-2.15	-3.48
	Phosphate-citrate	-3.39	-2.93
	Tris-citrate	-2.24	-1.68
	Average at pH 4	-2.65	-2.59
3	Glycine	-2.63	-3.12
	Citrate	-2.85	-3.53
	Succinate	-3.66	-2.52
	Phosphate-citrate	-3.93	-1.47
	Tris-citrate	-3.19	-2.67
	Average at pH 3	-3.25	-2.66

[a] Poliovirus at 1.8 x 10^9 particles per ml; reovirus at 1.2 x 10^9 particles per ml.

[b] All buffers were at 0.05M except phosphate-citrate and Tris-citrate which were mixtures of two components.

[c] $\log_{10}$ titer in acid minus $\log_{10}$ titer in PBS or Tris-buffered saline.

[d] EDTA, Ethylenediaminetetraacetate.

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Aggregation in Tris-citrate buffer (Fig. 38) was somewhat different than in phosphate-citrate or the more usual buffers (Fig. 36). The curve of poliovirus aggregation in Tris-citrate was also sigmoid in character, but it differed from the curve in phosphate-citrate due to the significant amount of aggregation at all pH values less than 7. This is also reflected in Table 4. Reovirus aggregation, on the other hand, demonstrated a linear plot with respect to aggregation versus pH in Tris-citrate. A least-squares analysis of the plot revealed a decrease of 0.7 $\log_{10}$ per pH unit, which resulted in a 5-fold decrease in the number of particles remaining in the top of the centrifuge tube for each 10-fold increase in hydrogen ion concentration.

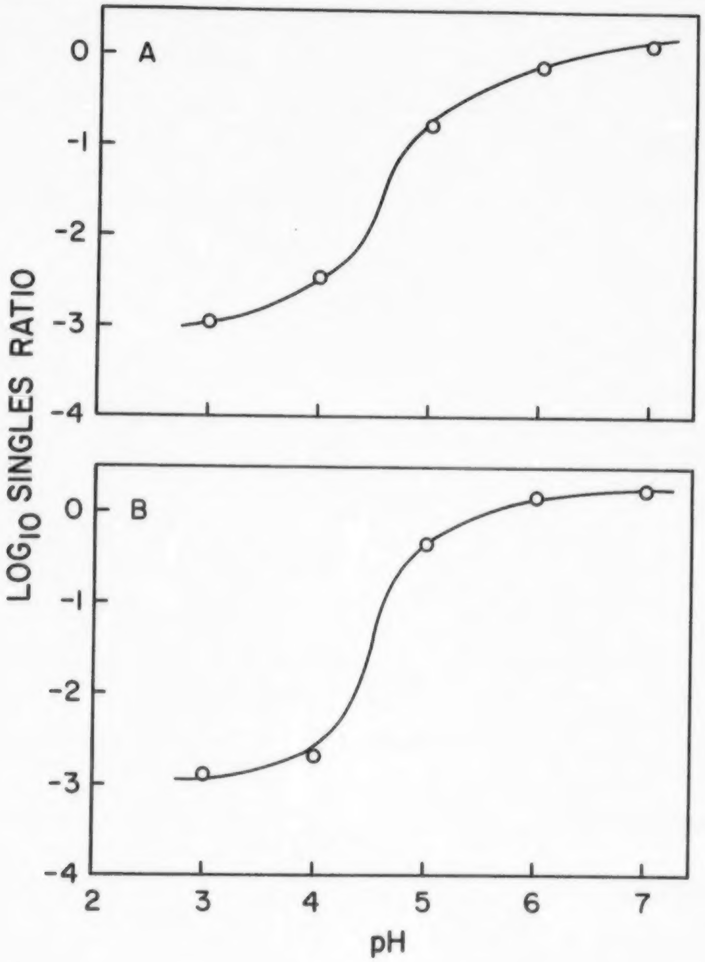

Figure 37. Aggregation of (A) poliovirus and (B) reovirus in McIlvaine phosphate-citrate buffer.

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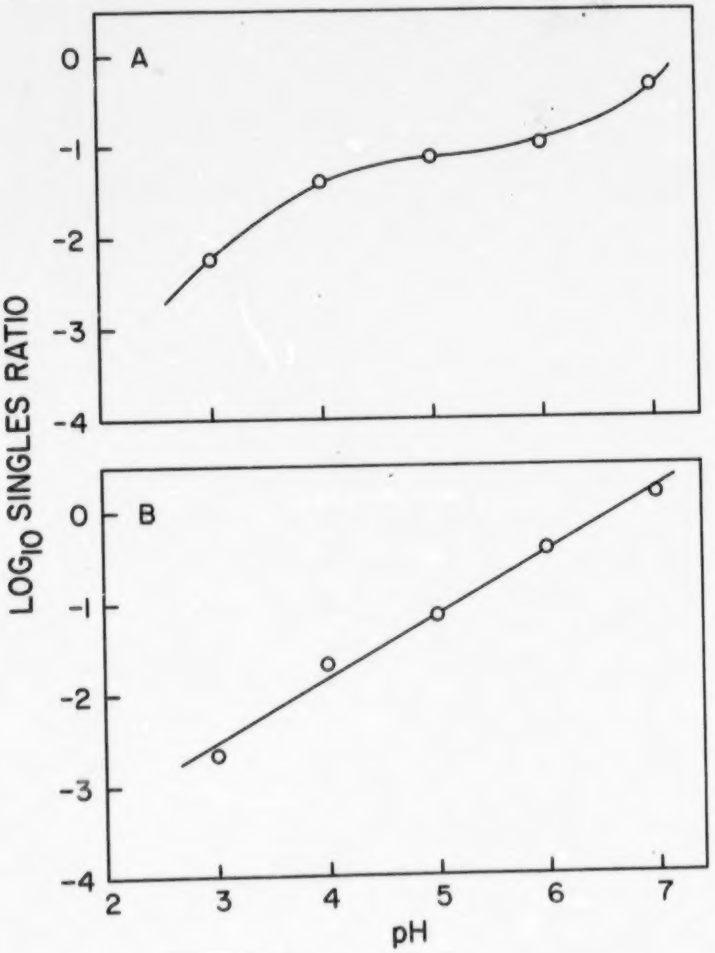

Figure 38. Aggregation of (A) poliovirus and (B) reovirus in Triscitrate buffer.

In contrast to the aggregation in acid buffers, neither poliovirus nor reovirus showed any tendency to aggregate in buffers in the alkaline range, with the exception of prominent aggregation of both viruses in borate buffer. Table 5 shows all of the buffers and pH levels tested for aggregation potential on poliovirus and reovirus. Further tests on borate induced aggregation of both viruses have revealed a complex form of aggregation which is at variance with the known fundamentals of aggregation of virus particles as induced by low-pH buffers (8), and will not be treated here. It should be noted, however, that, although borate buffer is widely utilized in the pH range 8 to 10, purified virus preparations may be induced to aggregate severely in borate suspension, whereas unpurified virus of comparable plaque-forming units per milliliter does not aggregate.

TABLE 5. Neutral and alkaline pH buffers tested for aggregation of of poliovirus and reovirus by a 4-h SPA test

Buffer[a]	pH range[b]	Aggregation of:	
		Poliovirus	Reovirus
Boric Acid-NAOH	8-10	Yes	Yes
Barbital-HCl	7.9-9.5	No	No
Glycine-NaOH	9-11	No (to pH 10.5)[c]	No (to pH 10.5)[c]
Tris-hydrochloride	7-9	No	No
Tris-citrate	7-9.5	No	No
Phosphate-carbonate	7-9	No	No

[a] Concentrations were 0.05M, except for Tris-citrate and phosphate-carbonate.

[b] Tested in 0.5 pH unit steps within the range specified.

[c] Viruses were inacivated at pH 11.0, and aggregation could not be determined.

SERIES VI. EFFECT OF IONIC ENVIRONMENT ON THE INACTIVATION OF POLIOVIRUS IN WATER BY CHLORINE (44)

Previous work has shown that poliovirus type 1 (Mahoney) remains dispersed as single particles in 0.05 M phosphate buffer at pH 7, but at pH 6 it tends to aggregate (7). This aggregation can be prevented by adding 0.1 M NaCl to the pH 6 buffer. To avoid any possibility of aggregate formation in this work, 0.1 M NaCl was included in pH 6 buffer as it was in previous work (22) and, for uniformity, in all the others as well. Accordingly phosphate-carbonate buffer (Table 1) was prepared with 0.1 M NaCl at pH 6, 7, 8, and 9, and virus was exposed in each of these buffers to 2.5 μM HOCl at 20C. This means that at pH 6, 7, 8, and 9, the concentrations of OCl^- present were 0.11, 0.90, 9.1, and 91 μM, respectively. In this series, pH 10 was omitted because the expected 900 μM OCl^- seemed impractical. The pH 10 experiments were made at lower chlorine concentration.

The progress of the inactivations can be seen in Fig. 39. There was a remarkable increase in reaction rate between pH 6 and 7 and successively smaller increases at pH 8 and 9, whereas the OCl^- concentration increased steadily by about a factor of 10 at each step. A second series of experiments was made like the first except that the 0.1 M NaCl was omitted from the buffer (Fig. 40). This reduced the ionic strength of the suspending fluids from 0.125 to 0.025, and it also reduced the reaction rate by a factor ranging between 2X and 4X, depending on the pH (compare Fig. 39). The effect of salt at pH 10 was sought by making experiments at a lower total free chlorine concentration (10μM) which will be in the form of OCl^-. Here the inactivation rate with 0.1 M NaCl present was of the order of 50 to 100 times faster than without it (Fig. 41). Pressing further with this extraordinary result, we observed that in the presence of the salt the inactivation rate of the virus increased with chlorine concentration only up to 10 μM; at 40 μM it went very little faster (Fig. 42). A similar saturation effect was observed in previously reported experiments (22) without salt. Under these conditions, the concentration for maximum rate was about 15 μM.

In the absence of 0.1 M NaCl at pH 10, the inactivation rate of virus was only about 1 $\log^{10}$ unit in 60 s (Fig. 41). Although the concentration of OCl^- was 4 times greater than that of HOCl at pH6 (Fig. 40) the inactivation rate was much slower. Apparently OCl^- is only about one-tenth as effective in the inactivation of this particular virus as HOCl. Still, at pH 7 where the OCl^- concentration was only 0.9 μM, the rate of inactivation was roughly three times as great as it was at pH 6 (Fig. 39). This must be due, at least in part, to a decrease in the resistance of the virus brought about by the NaCl. The further increases in rate at pH 8 and 9 may be attributable to the rapidly increasing OCl^- concentrations as well

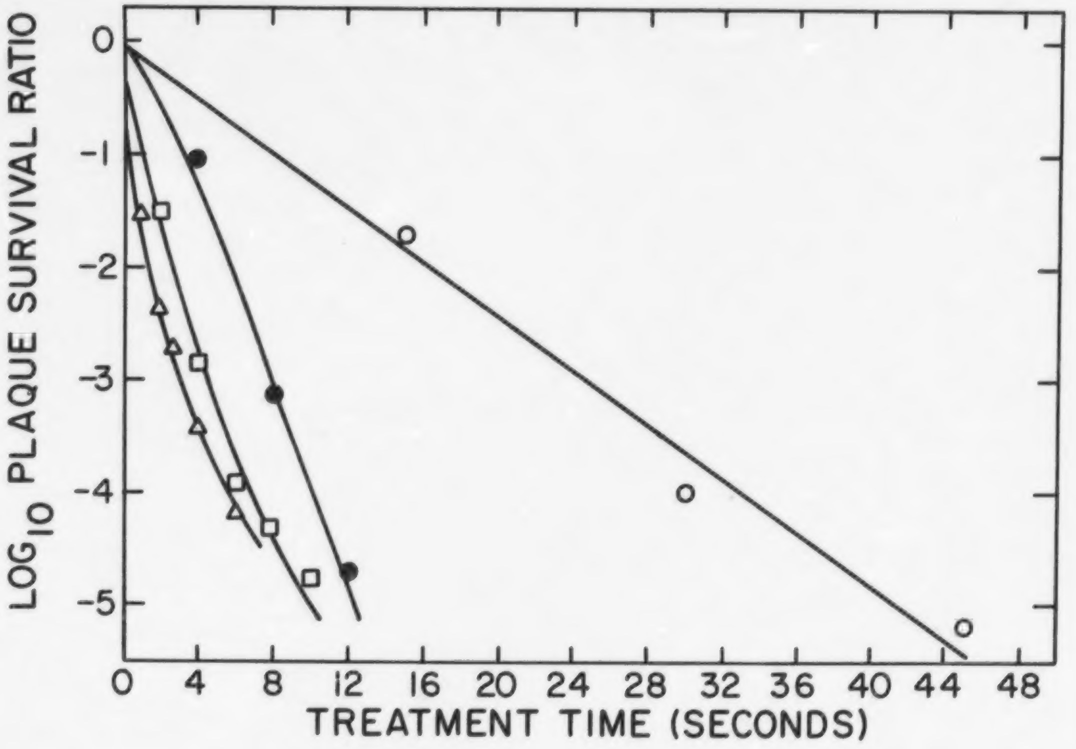

Figure 39. Inactivation of poliovirus (Mahoney) at 20°C in phosphate-carbonate buffer containing 0.1 M NACl by 2.5 μM HOCl and varying amounts of OCl^-, pH 6 (circles), pH 7 (filled circles), pH 8 (squares) and pH 9 (triangles).

as the increased sensitivity of the virus to OCl^- in the presence of salt and may involve the saturation effect observed with OCl^- at pH (Fig. 42) as well.

Sodium chloride added to the buffer at pH 6 prevented aggregation, although in 0.05M phosphage buffer at pH 6 this aggregation was quite slow (8). To get the reaction rate of chlorine on the dispersed virus without salt, experiments were made that showed that it could be done even at pH 6 in the inactivation experiment was done within a few minutes of the time the dispersed stock virus at pH 7 was diluted and adjusted to pH6. This is the method by which the top line of Fig. 39 was obtained and also the "no salt" line of Fig. 43. Figure 43 also shows the slower chlorine inactivation rate that was obtained if the virus was permitted to aggregate before the run was made. In addition, it shows that the accelerated rate produced by 0.2 M NaCl is little, if any, more than that caused by the 0.1 M salt.

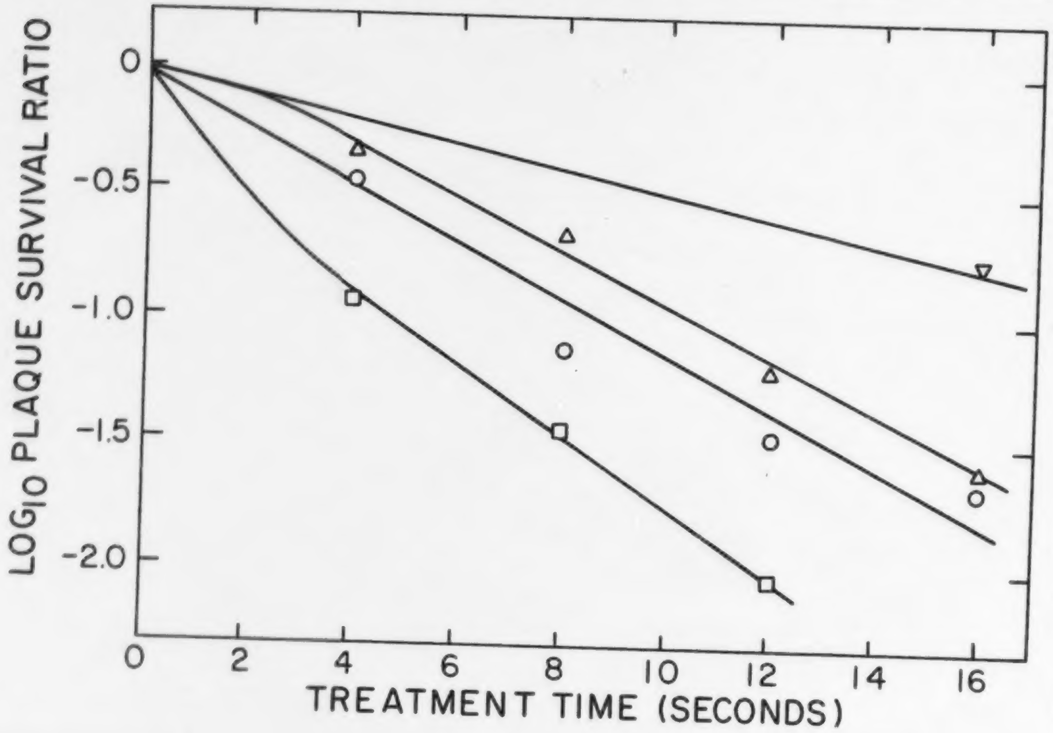

Figure 40. Inactivation of poliovirus (Mahoney) at 20°C in phosphate-carbonate buffer same as Fig. 39 but without NaCl. Lines from top to bottom are for pH 6, 7, 8, and 9 respectively.

The plaque titer of our purified preparations of poliovirus has not declined to any measurable extent on standing 4 h in water, at 25°C in the pH range 3 to 10, in any of several buffer systems, or in phosphate or tris (hydroxymethyl) aminomethane-buffered saline. Others have reported the pH range of maximum heat stability to be pH 3 (45,46). Thermal degradation occurs more rapidly in the alkaline region, and it is augmented in the presence of sodium chloride particularly at the higher pH's (45), but we are not aware of any previous report showing that chlorine destroys viruses more rapdily when NaCl is present. This should be important in seawater.

Free chlorine inactivated this virus under all conditions that we have examined, but it is clear that when no NaCl was added to the buffer, a given concentration was much less effective at pH 10 than at pH 6. Now the complexity of the situation becomes apparent; the partition of the free chlorine between HOCl and OCl depends primarily on the pH and, to a lesser extent, on the salt concentration, and the sensitivity of the virus varies also with pH and with the amount of salt present. Added to this is the dependence of virion aggregation on both pH and salt concentration.

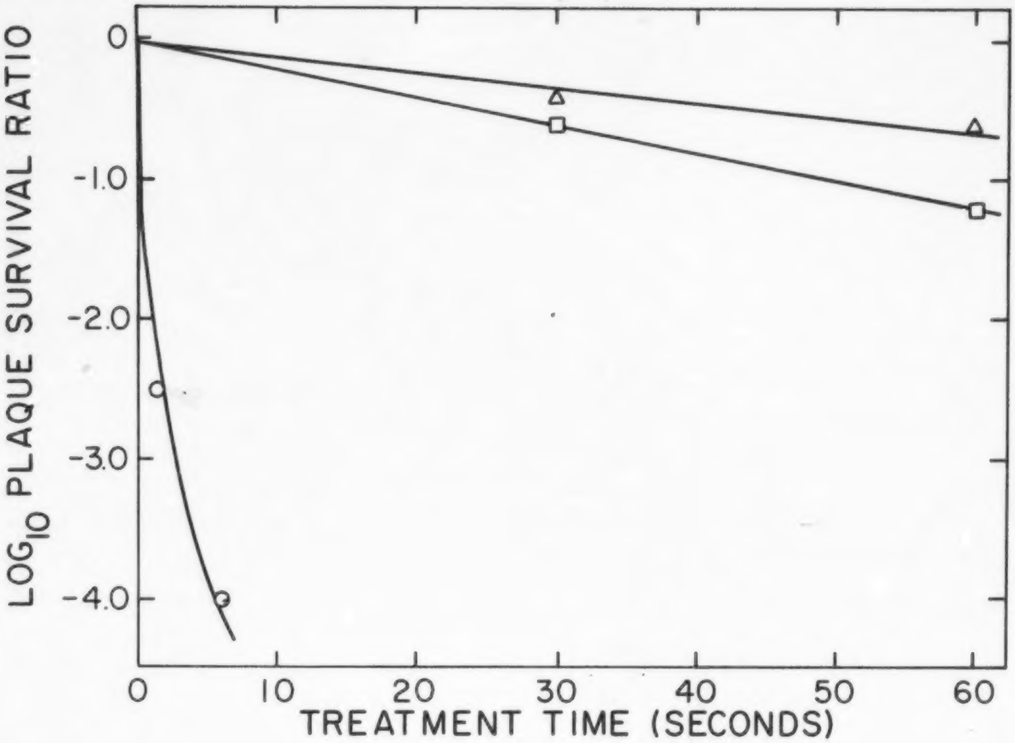

Figure 41. Inactivation of poliovirus at 20°C by 10 μM chlorine at pH 10 without buffer or salt (squares), in dilute phosphate-carbonate buffer (triangles) and in the same buffer containing 0.1 M NaCl (circles).

It is clearly not a simple matter to determine the relative effectiveness of HOCl and OCl^- in the disinfection of poliovirus, but an initial step would seem to be to perform experiments at pH 6, 7, 8, 9, 10, keeping the concentration of HOCl consistant. This was done at 2.5 μM HOCl, at pH 6, 7, 8, 9, where the corresponding concentrations of OCl^- are calculated to be 0.11, 0.90, 9.1, and 91 μM respectively. At pH 10, we reduce the total free chlorine to 10 μM to keep it within a practical range. Calculations of relative amounts of HOCl and OCl^- were made by the method of Davies (47) as applied by Morris (48) and by Sugam and Helz (49). The acid ionization constants of HOCl given by Morris (48) were used.

In the absence of NaCl at pH 10, where essentially all of the 10 μM free chlorine (Fig. 41) is OCl^-, approximately 10% of the plaque titer survived 60 s of exposure. At pH 6, 2.5 μM HOCl reduced the survival ratio to approximately the same level in 16 s. It appears from this alone that OCl^- is poor compared with HOCl as a disinfecting agent. When NaCl was present, there was a 50 to 100 X increase in virus sensitivity at pH 10 where the reacting species was OCl^-, compared with an increased sensitivity at pH 6 of threefold, where the reacting species was HOCl. It was not

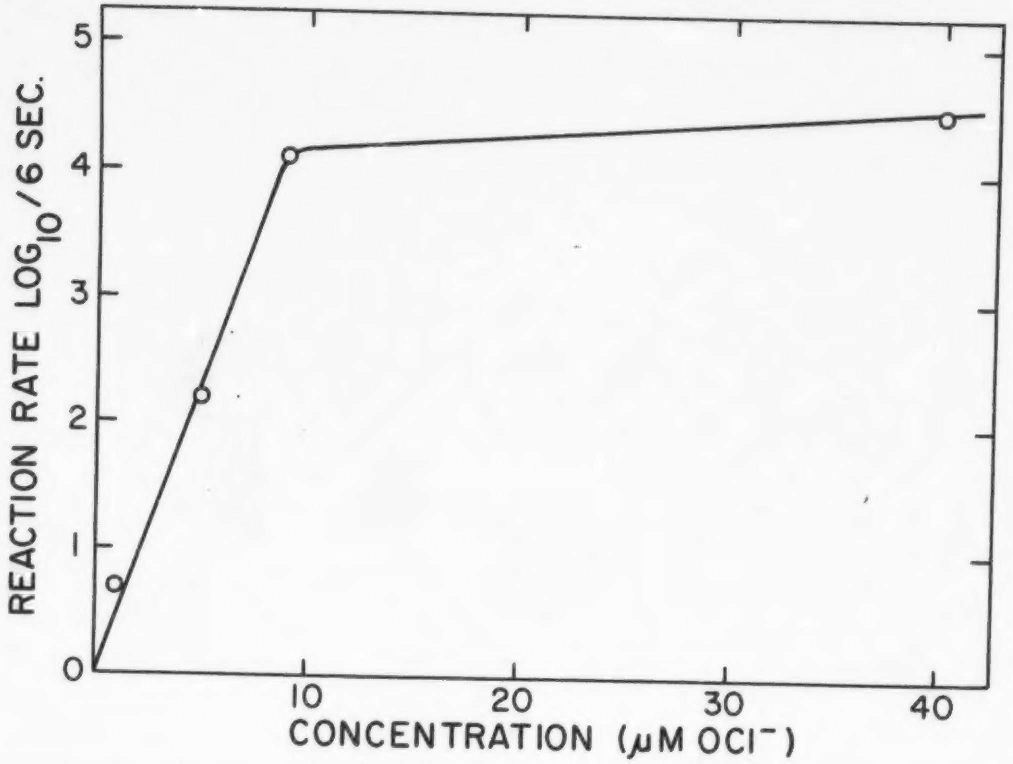

Figure 42. Inactivation rate of poliovirus at 20°C in dilute buffer containing 0.1 M NaCl as a function of OCl^- concentration.

clear from these results whether the increased sensitivity to OCl^- in the presence of NaCl extends to the lower pH values of 6 and 7. If it does, the inactivation rate for 1 μM OCl^- at pH 10 in the presence of NaCl (42) still does not appear to account completely for the increases from 0.11 to 0.9 μM. It is possible that the virus becomes more sensitive to the 2.5 μM HOCl with increasing pH, a trend that may continue at pH 8 and 9.

The effect of added salt (0.1 M NaCl) on the rate of degradation of plaque titer by HOCl can be seen by comparing Figs. 39 and 40 to be about a threefold increase at pH 6. In this case, the virus will aggregate without the salt (6) so the inactivation experiments were performed quickly, before appreciable aggregation could occur (8). The much greater increase in inactivation rate of chlorine produced by salt at pH 10 might be suggested but not predicted in view of the similar effect that has been observed (45) with NaCl on the thermal degradation of this virus. It is, nevertheless, a fact whose practical significance cannot be ignored, for it is apparent that, at high pH, OCl^- destroys this poliovirus more rapidly in the presence of 0.1 M NaCl than HOCl does at pH 6 in the same buffer with or without the added salt.

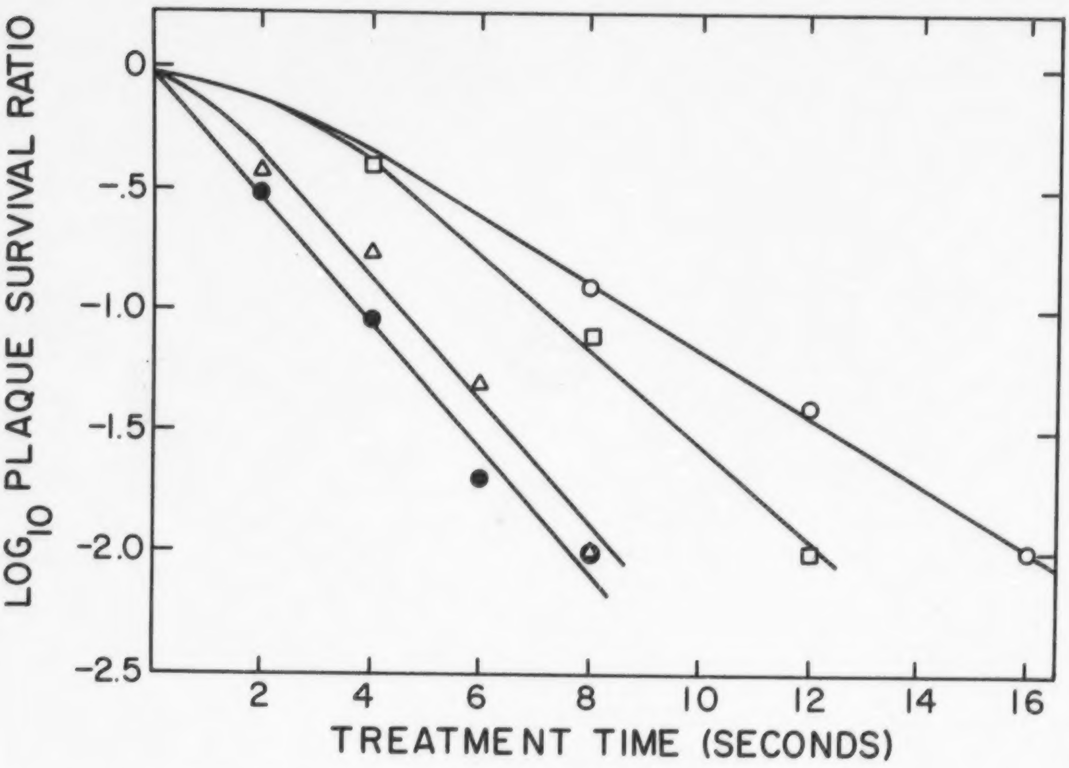

Figure 43. Inactivation of poliovirus by 20 μM HOCl at 20°C in dilute phosphate-carbonate pH 6 buffer, 5 minutes after dilution (squares), one hour after dilution (circles), in buffer containing 0.1 M NaCl (triangles) and with 0.2 M NaCl (filled circles).

At very low ionic strength (0.00001, pH 10 without buffer) plaque titer was reduced more rapidly by 10 μM OCl^- than in phosphate-carbonate buffer of ionic strength 0.027 and the same pH (Fig. 41), although the difference is not very great. This suggests that the greatly increased rate at pH 10 in the presence of 0.1 M NaCl may be a specific effect of the sodium or chloride ions. Potassium chloride, included in borate buffer at pH 10 by Scarpio et al. (23), yielded a more rapid poliovirus (Mahoney) inactivation by chlorine than they observed at pH 6. This may mean that KCl and NaCl act similarly, but the comparison cannot be made accurately because it is not clearly stated in their paper what concentration of KCl was used, and no attempt was made in their experiments to control virion aggregation which is not always negligible (Fig. 43). The most effective concentration of NaCl seems to have been approached at 0.2 M, since the reaction rate with 0.2 M (Fig. 43) is very little greater. Other salts and other viruses had not been tested in this respect, at this time but subsequent work indicates that the reaction rates of chlorine with coxsackie B5 at pH 10 are increased not only by NaCl but also by KCl and by CsCl. Doubtless other salts commonly found in hard waters should be investigated as well.

The ability of OCl^- in the presence of 0.1 M NaCl to inactivate poliovirus (Mahoney), is subject to similar limitations that have been observed (4) in the data of Weidenkopf (16). After increasing linearly to 10 µM, the rate increased very little at 40 µM (Fig. 42).

Finally, it has become clear from the results of recent work on viral disinfection (3,4,46) that many more variables are present than were previously suspected (16,18). Therefore, we strongly urge that in the future, all workers in viral disinfection control for and clearly state in the published paper the following variables: (i) the time of contact of the virus with the disinfectant, (ii) the temperature of the reaction, (iii) the total ionic strength of the reaction mixture, (iv) the pH of the reaction mixture, (v) the type of buffer used to maintain the pH, (vi) the total chlorine concentration and the relative amounts of HOCl and OCl^-, and (vii) the state of aggregation of the virus. Only when all of these parameters are known will it be possible to make adequate comparisons between data from different laboratories.

SERIES VII. INACTIVATION OF POLIOVIRUS 1 (Brunhilde) BY CHLORINE IN WATER (50)

All of the inactivation data presented so far for poliovirus has been obtained with the Mahoney virus. At this point, it appeared desirable to compare the chlorine resistance of the Mahoney virus with the closely related Brunhilde virus. All the experimental conditions were the same and the progress of inactivation of well dispersed Brunhilde virus by several different concentrations of HOCl at 20°C in dilute phosphate-carbonate buffer at pH 6 is shown in Fig. 44. The stock virus was diluted 60x with phosphate-carbonate buffer, and experiments were done within 5 min of the time of dilution. The log survival ratio was essentially a linear function of reaction time. The inactivation rates did increase continuously but not in a precisely linear fashion with the HOCl concentration (Fig. 45), and there was no indication that a maximum had been reached at a 40 μm concentration of HOCl.

When 0.1 M NaCl was included in the dilute phosphate buffer, the inactivation rate was doubled (Fig. 46), but it was still not as fast as the inactivation rate of poliovirus (Mahoney) under similar condnitions (22), and it is worthy of note that no transient departures from linearity were seen during the first 20 s of contact with the chlorine either with or without the salt.

At pH 10, where the active disinfecting agent was OCl^-, the rate of reduction in plaque-forming units (PFU) in dilute buffer alone (Fig. 47) was only about one-fifth as fast as it was at pH 6, but the effect of salt was much greater. The time required to inactivate 99% of the PFU without 0.1 M NaCl was 31 times greater than when the salt was present. This means that disinfection was faster at pH 10 with salt present than it was at pH 6 whether salt was present or not.

The possibility that aggregation of the virions might exert an influence on these reaction rates at one or both pH values was examined in detail. Electron micrographs of the virus deposited on collodion films (KA method) were made on preparations at pH 7. There was no evidence of aggregation, although the virion concentration was high (1.3 X 10^{12}/ml). The stock virus at pH 7 was used as the dispersed control for a series of sedimentation tests (8) that were made at other pH values (48). At pH's 7, 8, and 9 no aggregation was detected. At pH 10 there was some loss in plaque titer, but this was found to be a loss of infectivity rather than sedimentaion of supernatant PFU due to virion aggregation. At pH 6 aggregation set in. It was slow when 0.1 M NaCl was present, but in the absence of salt only about 10% of the population remained as single particles after 4 h at 25°C in the dilute buffer used for inactivation experiment. All inactivation experiments at pH 6 were made within 5 min of

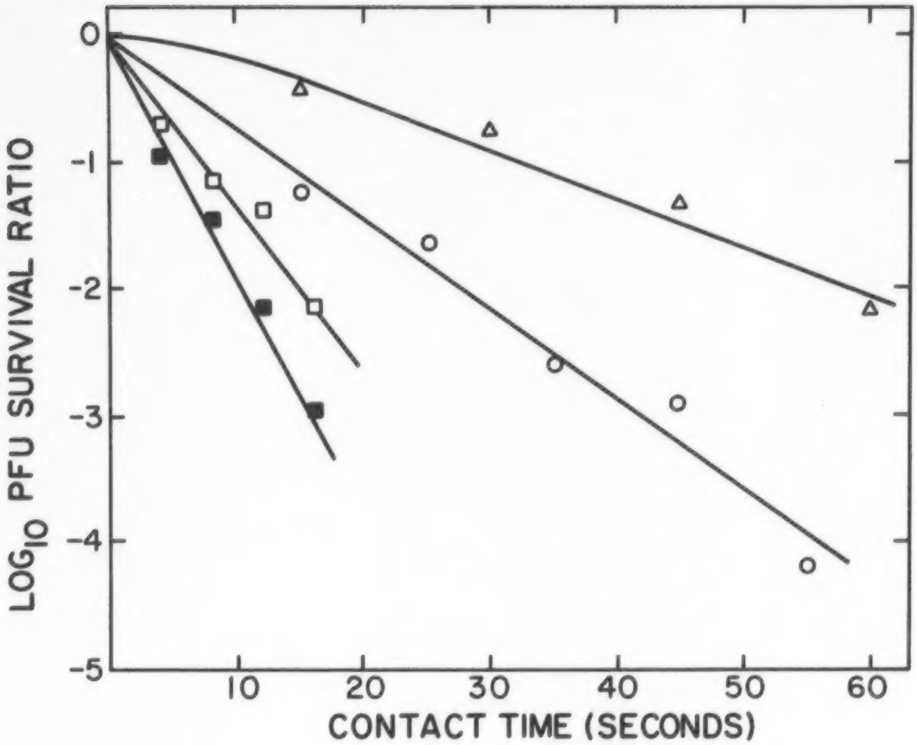

Figure 44. Inactivation of poliovirus (Brunhilde) at 20°C by HOCl, 10 µM (triangles), 20 µM (circles), 30 µM (squares) and 40 µM (filled squares).

a 60-fold dilution of the stock virus in pH 6 buffer, so very little aggregation could have occurred. At pH 5 aggregation was rapid and even more so at pH's 4 and 3. At pH 3 only 1 in 1,000 of the original single particles remained when on salt was added to the buffer (Fig. 48). This acid aggregation profile is very similar to those of both reovirus and poliovirus (Mahoney), for which the isoelectric points have been found at pH's 3.7 and 8.2 respectively.

The isoelectric point of the Brunhilde strain of poliovirus was measured in this work by the same technique as that used in previous work with the Mahoney virus. Figure 49 shows one major peak of PFU at pH 7.3 and small, probably insignificant, amounts scattered as far as pH 9.5.

The inactivation kinetics shown in Fig. 44 reveal no transient departure from linearity such as that so prominently displayed by echovirus (24) and poliovirus LSc (51). Three of the lines are remarkably straight down to survival levels of 10^{-2} to 10^{-4}, the only departure being the slight lag in the top line. It is unlikely that this is caused by virion aggregation. Although it is true that Fig. 48 indicates the onset of colloidal instability at pH 6, the time between the dilution of the stock

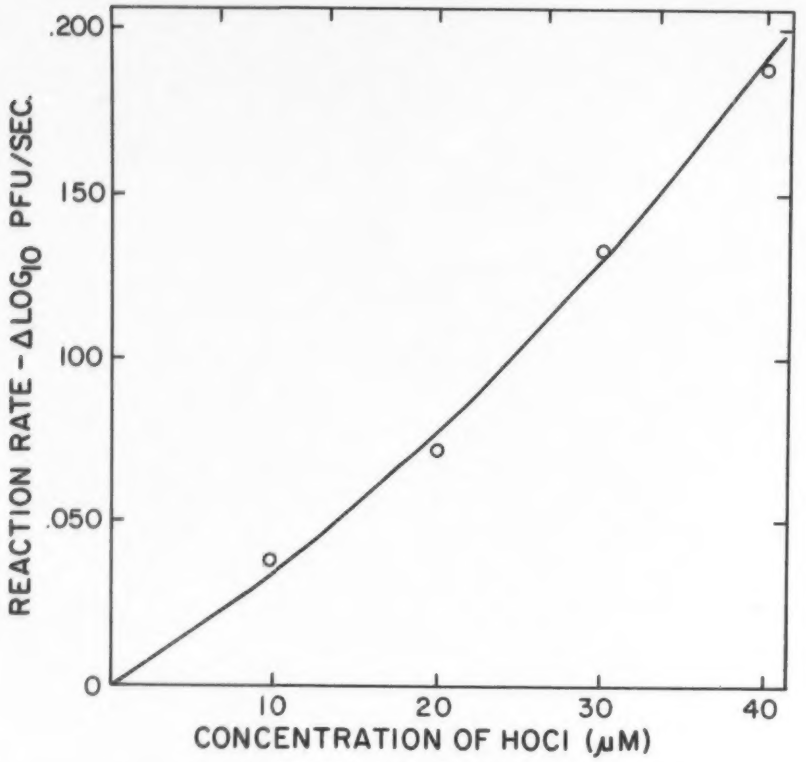

Figure 45. Inactivation of poliovirus (Brunhilde) at 20°C as a function of HOCl concentration.

virus in the pH 6 buffer and inactivation experiment was not sufficient for aggregation; it can occur only if most of the virions are aggregated (52).

The steady increase in the disinfection rate with HOCl concentration (Fig. 45) distinguishes the Brunhilde strain from the Mahoney strain of poliovirus because the latter increases only very slowly with concentrations greater than 10 μM (16,22). Like the Mahoney strain, the Brunhilde strain showed no tendency to aggregate in dilute buffers at its isoelectric point, which was revealed by electrophoretic focussing experiments to be at pH 7.3 (Fig. 49). Neither aggregated at or above pH 7, but both aggregated slowly at pH 6 and increasingly as pH was decreased to 5 and further into the acid range.

Inasmuch as virion aggregation is not likely to influence the inactivation experiments reported here, the straight line characteristics of most of the graphs might be attributed to the use of dispersed virus were it not for the fact that equally well dispersed Mahoney strain poliovirus produced predominantly curved lines in experiments of exactly the same kind. These results indicate that a departure from first-order

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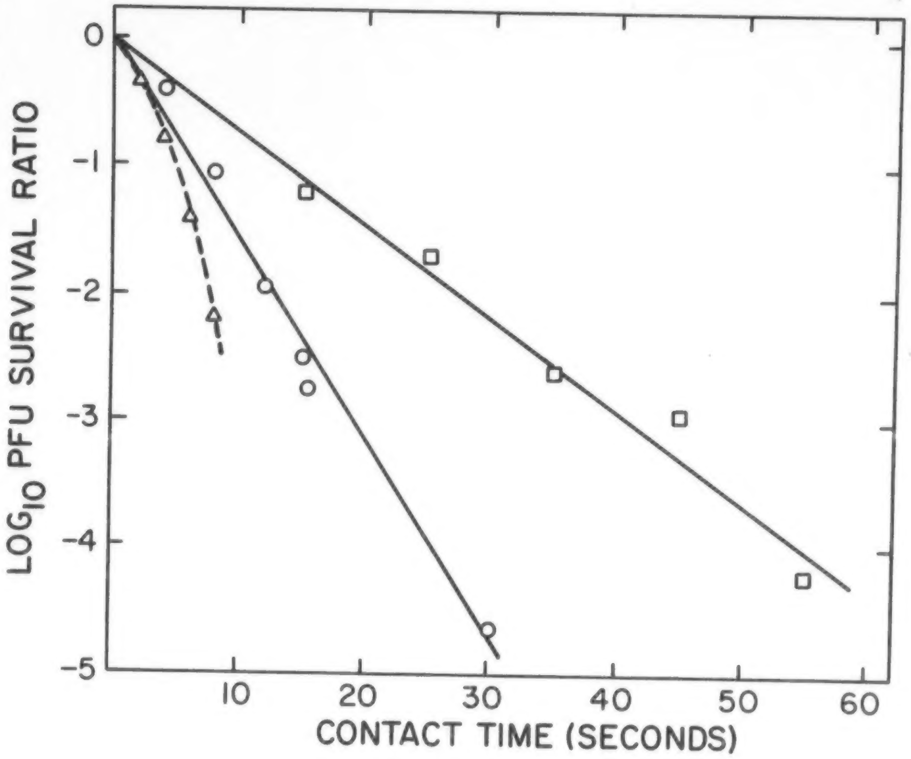


Figure 46. Effect of NaCl on the inactivation of poliovirus (Brunhilde) by HOCl. Virus in dilute buffer (squares), dilute buffer containing 0.1 M NaCl (circles) and Mahoney virus for comparison (triangles).

kinetics of the inactivation of these viruses by chlorine is not necessarily caused by aggregation among the virions. Other influences, presumably related to the mechanism of the reaction of chlorine with individual virions, have thus been revealed.

One characteristic of the Brunhilde strain is outstanding. In the presence of the relatively weak disinfecting OCl^- form of chlorine at pH 10, the addition of 0.1 M NaCl increased the reaction rate 31-fold (Fig. 47). This was not a deaggregating effect of salt; there was no aggregation of the virions at pH 10, yet the time required to reduce the PFU survivial ratio to 10^{-2} was reduced from 127 s to less than 4 s, which is less than the time required by the same 20 μM chlorine in the form of HOCl at pH 6. This effect was observed earlier with the Mahoney strain as well, and there the rate differential was even greater. Although it is not at all clear why the presence of the additional Na^+ and Cl^- ions should so alter the reactivity of the OCl^-, it may be pertinent to recall the work of Salo and Cliver (46), who showed that the thermal stability of several picornaviruses was much greater in acid than in alkaline conditions. An

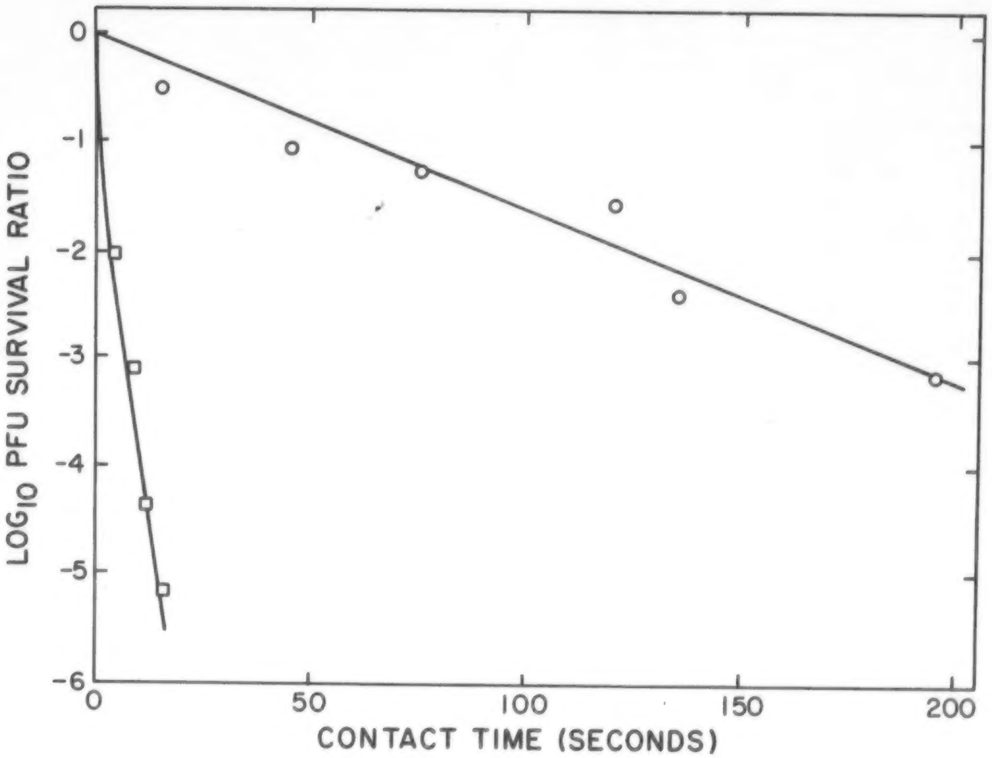


Figure 47. Inactivation of poliovirus (Brunhilde) at 20°C by OCl^- at pH 10 in dilute phosphate-carbonate buffer without added NaCl (circles) and with 0.1 M NaCl added (squares).

increase in ionic strength along with high pH may lossen or otherwise weaken the protective covering capsid of the virion. Whatever the mechanism, the facts revealed are of immediate practical significance because the weak disinfection action of OCl^- previously reported by us and others (22) can apparently be augmented 30-150-fold if NaCl is added. This effect must appear in experiments involving the chlorination of viruses in seawater, but we are not aware of any publication of such results. Various hard waters containing salts of several kinds are likely to show salt effects of this kind also.

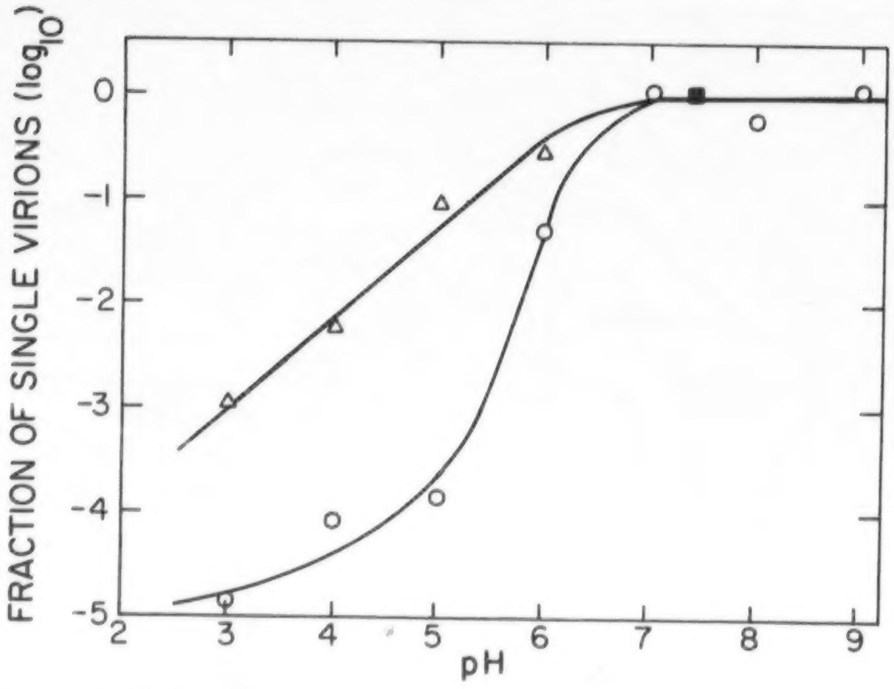


Figure 48. Aggregation of poliovirus (Brunhilde) at 25°C as a function of pH, when 0.1 M NaCl was added to the buffer (triangles) and when it was not (circles).

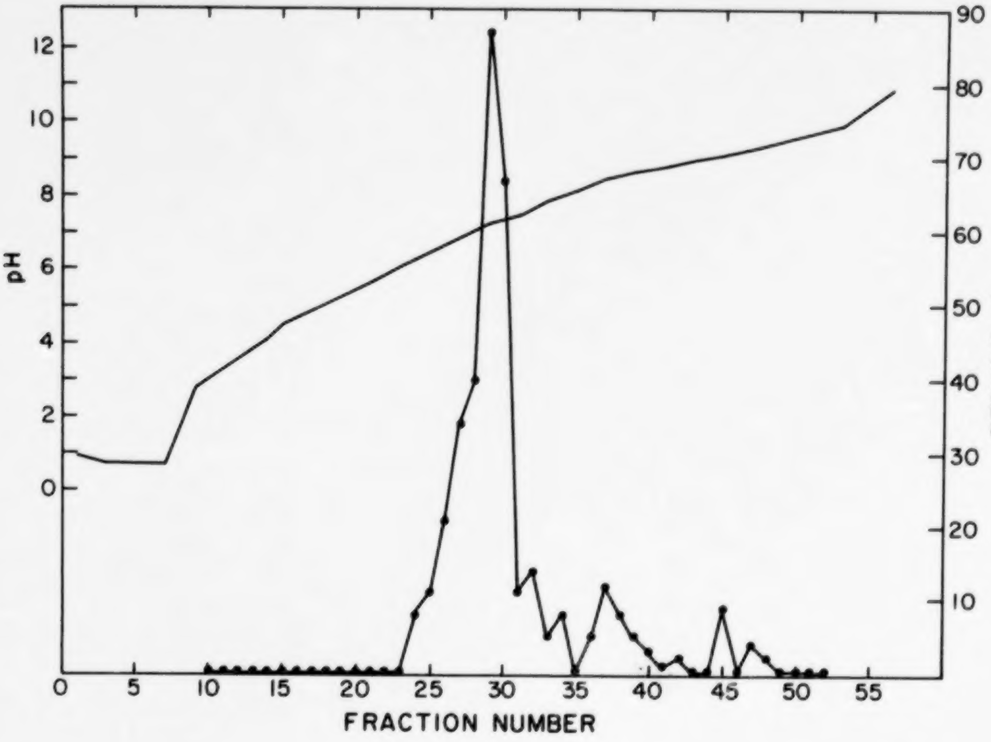


Figure 49. A major peak of infectivity of poliovirus (Brunhilde) proved to be isoelectric at pH 7.3.

SERIES VIII. INACTIVATION OF COXSACKIEVIRUSES B5 AND B3 BY CHLORINE IN WATER (53)

Coxsackie virus B5 was prepared, purified and assayed by plaque formation on monolayer cultures of HEp-2 cells but the purified virus invariably became aggregated, usually in the first few hours but always after 3 days of storage under the same conditions that preserved the poliovirus in well-dispersed condition. Virion aggregation test (VAT) showed 74 to 100% of the PFU were aggregates of at least 18 virions. Similarly prepared poliovirus routinely gave only 0-5% of such aggregates by the same test. When VAT was performed on crude (unpurified) CB5 virus there was some aggregation (11 to 39% in the large aggregate category), but much less than that of the purified virus. Occasional preparations of virus, if tested by the virion aggregation test immediately after removal from the sucrose density gradient, would show little or no aggregation, but none remained in this condition as long as 3 days and most were aggregated by the time they could be tested.

Electron micrographs made from kinetic attachment preparations of freshly purified virus showed very few aggregates and fewer virions than expected considering the ample plaque titer of this freshly purified virus. Virus particle counts of 3 X 10^{10} to 8 X 10^{10} were calculated from electron microscope pictures of several such preparations, but these were thought to be low because both poliovirus (Mahoney) and echovirus (Farouk) yielded many more particles for comparable plaque titer. After reading the paper by Totsuka et al. (54), we added 100 μg of diethylaminoethyl dextran of 500,000 molecular weight (Pharmacia, Uppsala, Sweden) per ml of 0.05 M phosphate buffer (pH 7) in the sucrose gradient and produced purified CB5 without appreciable aggregation. This was verified by the virion aggregation test in which only 2% of the PFU were found in the large aggregate region as well as by electron microscopy. Unfortunately, this well-dispersed virus could not be used in disinfection experiments because of the excessive chlorine demand of even this small amount of diethylaminoethyl-dextran. Attempts to do so did, however, reveal an increased inactivation rate in spite of simultaneous loss in free chlorine.

Electron micrographs showning highly aggregated CB5 were never obtained when the kinetic attachment method of preparation was used, but large aggregates were seen when purified virus was diluted 10X with phosphate-buffered saline and the virions sedimented upon agar by the pseudoreplica method (1).

Electrophoretic focusing of CB5 was done,as it was with the poliovirus, in a sucrose gradient containing initially the same amount of virus in all parts and an ampholyte mixture covering the pH range 3 to 10 in an LKB APPARATUS. After focusing there was a broad peak containing 69% of the PFU in the region of pH 3.5 to 4.1; the rest was scattered, with little evidence of peaks, as far as pH 9. There was no significant peak at or above pH 7.

When CB5 was prepared in a sucrose gradient containing 100 μg of diethylamino-ethyl dextran per ml, there was no aggregaton as shown by electron microscopy and, on electrophoresis, the PFU accumulated in two places (Fig. 50). A low broad peak appeared in the pH 3 to 4 region just as it did with untreated virus, but a second, sharper peak appeared at pH 7.2.

Inactivation of the CB5 at pH 6 and 20°C by several different concentrations of HOCl (10, 15, 20, and 30 μM has shown very little evidence of the departure from first-order kinetics (Fig. 51) that might have been expected in such experiments with aggregation virus. The slopes of the lines of Fig. 51 yield the reaction rates plotted in Fig. 52 which increase with HOCl concentration in a linear fashion except that a fifth experiment (not shown on Fig. 51) at 37 μM HOCl indicated that a maximum had been reached with not further increase above 30 μM HOCl.

Careful attention to the inactivation kinetics during the first 10 s of reaction with 20 μM HOCl has revealed a curious transient when 0.1 M NaCl was present. This salt was used in previous experiments with poliovirus to prevent aggregation. It had no effect on the aggregaton of CB5, but when it was present in chlorine experiments the plaque titer dropped immediately (within 2 s) by 1.3 logs (20X). Figure 53a shows all the data from several experiments which show a 15-s period of delay and suggest a slight increase before falling into a linear decline. We have no evidence of such transient changes in reaction rate when there was not NaCl present, and the linear inactivation rate was about the same after the first 10 s whether there was 0.1 NaCl present or not (Fig. 53b). When CsCl was substituted for NaCl the reaction rate was semilog linear with about the same slope. No attempt was made to detect short-time transients with CsCl.

At pH 10, 20°C, and 20 μM OCl^-, the inactivation rate (Fig. 54) was much slower than at pH 6. When 0.1 M NaCl was added to the pH 10 buffer, the time for 99% plaque reduction was reduced from 7.9 min to 17 s. The effects of 0.1 M CsCl and 0.1 M KCl were similar to that of NaCl except in magnitude (Fig. 54). The cesium salt was much less effective in reducing inactivation time than the sodium salt, and the KCl was more effective.

Finally, inactivation experiments were made at 7 μM HOCl and 5°C for purposes of direct comparison with the experiments of Engelbrecht et al. (55). Again, the inactivation rate was essentially the same whether NaCl was present or not, but when it was present there was an initial 1-log plaque titer drop followed by a slight rise and subsequently by a slow steady drop beginning at 60 s (Fig. 55). This behavior is somewhat like that seen with the higher concentration of HOCl (Fig. 53), but it takes place more slowly. The transient irregularity was not present when the

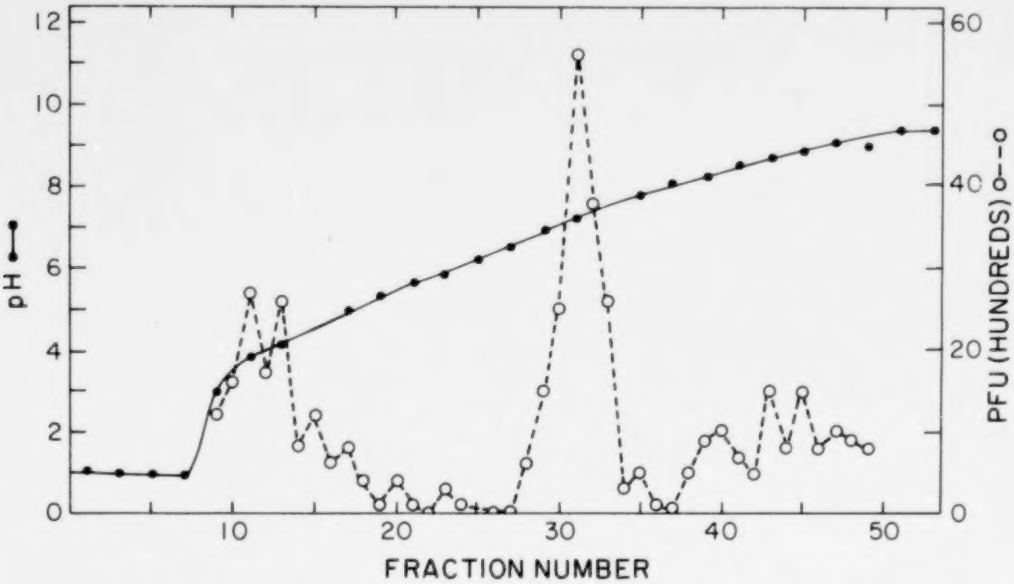
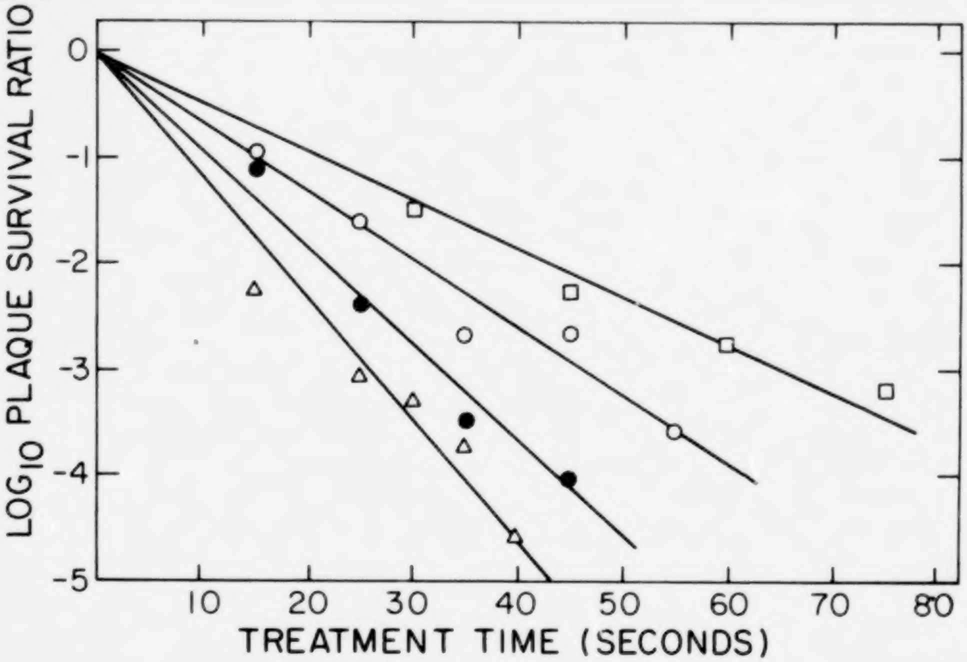


Figure 50. Electrofocusing diagram of PFU of soxsackievirus B5 in the presence of 100 μg/ml DEAE dextran which was necessary to prevent virion aggregation.



Fgiure 51. Inactivation of coxsackievirus B5 at pH6 and 20°C by HOCl, 10 μM (squares), 15 μM (circles), 20 μM (filled circles) and 30 μM (triangles).

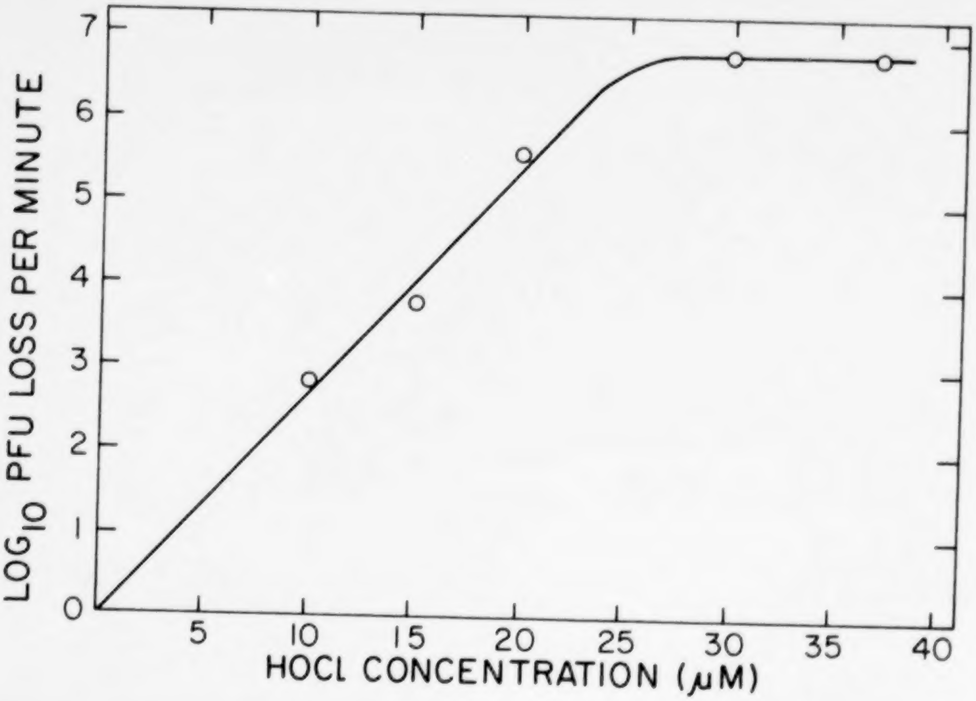


Figure 52. Inactivation rate of coxsackievirus B5 at 20°C as a function of HOCl concentration.

NaCl was omitted. The HOCl data from the Engelbrecht paper (55) are indicated by the dotted line on the graph (Fig. 55) where the time for 99% inactivation was only slightly longer (39%) than that shown by our data.

The behavior of CB3 was in some respects quite different from that of CB5 especially with regard to aggregation. Although preparation, purification, and storage of the two viruses were the same, electron microscopy, virion aggregation tests and single particle analyses have shown no evidence of virion aggregation of CB3 in 0.05 M buffers from pH 6 to 8.2. Below pH 5 purified preparations of this virus aggregated rapidly (Fig. 56). This closely resembles the behavior of echovirus (24) in the acid region.

The inactivation rate of CB3 at pH 6 by HOCl in the absence of NaCl is shown in Fig. 57. When 0.1 M NaCl was added, there was no significant change in either the initial transient or final steady inactivation rate.

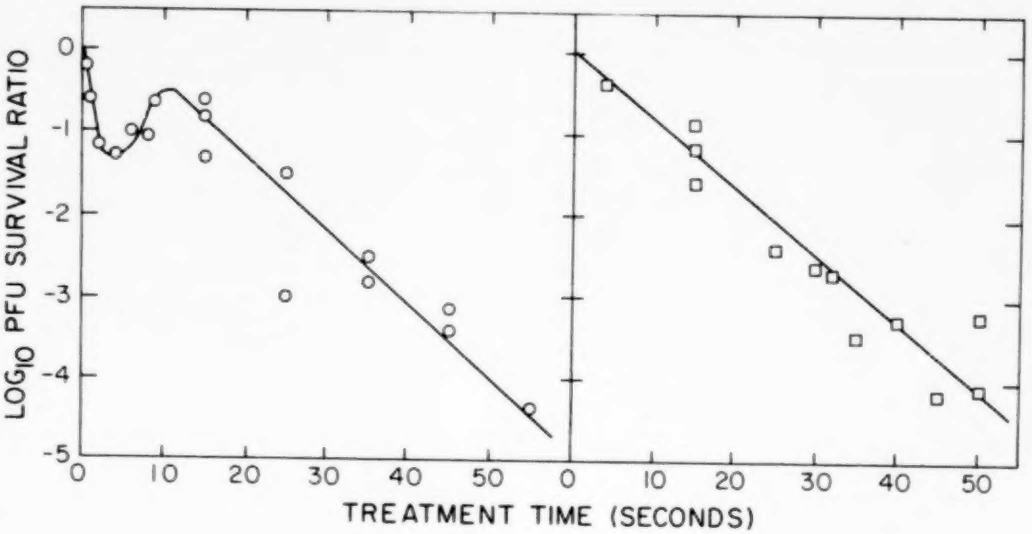


Figure 53. Inactivation of coxsackievirus B5 by 20 μM HOCl (A) when 0.1 M NaCl was present in the pH 6 buffer and (B) when it was not.

At pH 10 the inactivation of CB3 was very slow (Fig. 58). At 20°C in 20 μM free chlorine (OCl^-), the PFU survival ratio of 10^{-4} was reached in 15.5 min. When 0.1 M NaCl was added to the buffer, the time was reduced to 25.5 s. Here again, as with CB5 the presence of 0.1 M NaCl at pH 10 made a major increase in the inactivation rate by OCl^-.

Sugam and Helz (49) have observed significant ion pairing in seawater, and they have suggested that this might increase the intoxicity of chlorinated waters at pH greater than 8.0 With this in mind we determined the extent of ion pairing for three concentrations of KCl and NaCl in water containing approximately 200 μM OCl^- at pH 10. Solutions of 0.50 M KCl and 0.50 M NaCl first were made chlorine demand-free. Then suitable dilutions in chlorine demand-free water were made to obtain 0.05, 0.10 and 0.50 M solutions to which 0.05 M NaOCl was added, and the pH was adjusted with dilute NaOH to botain approximately 200 μM OCl^-. These chlorinated salt solutions were tested in a Cary model 219 scanning spectrophotometer to determine the peak of absorance at 292 nm. The total chlorine concentration also was determined by amperometric titration. From these

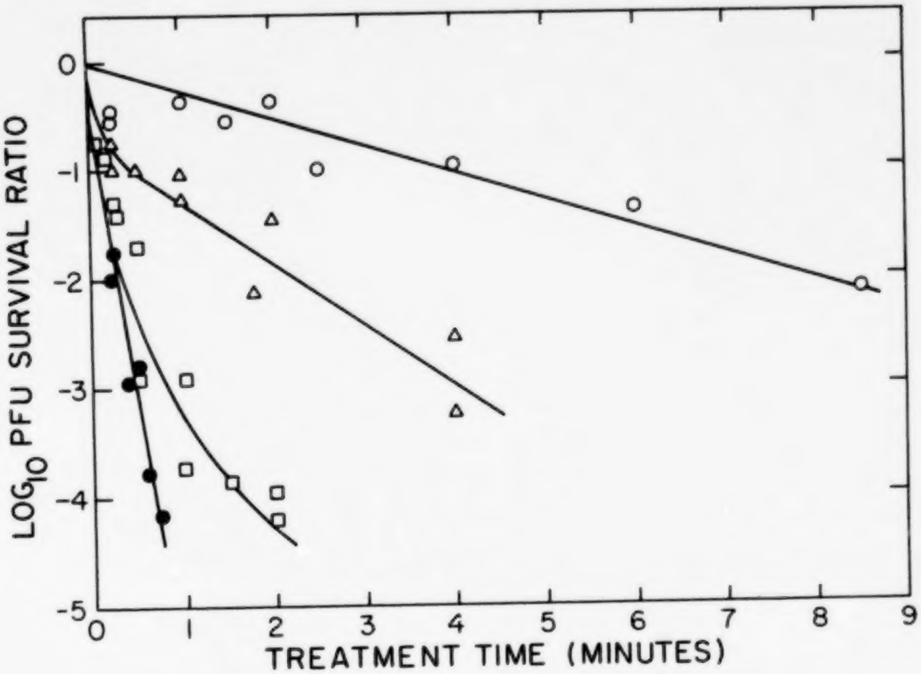


Figure 54. Inactivation of coxsackievirus B5 by 20 μM OCl^- at 20°C with 0.1 M KCl in the buffer (filled circles), with 0.1 M NaCl (squares), with 0.1 M CsCl (triangles) and with no salt added (circles).

data the molar absorptivities (ε) at 292 nm were calculated from Beer's law for OCl^- in solution with various concentrations of Na^+K^+. The calculated extent of ion pair fromation between OCl^- and these cations can be seen in Table 6, where the ε values for the salt solutions are compared with the expected value of ε 350 for free OCl^- at its absorbance maximum. These calculations are based on the assumption that paired OCl^- ions have a much lower molar absorptivity than unpaired OCl^- ions. Table 6 shows that the potassium ion is slightly better at pair formation than sodium, particularly at the low concentrations.

If enough facts relevant to the inactivation of viruses by chlorine in water are to be assembled to provide a basis for the generalization that is needed by those who must make reused water safe for drinking, many viruses must be examined. Of the two viruses chosen here, one (CB5) has high chlorine resistance (55), and the other (CB3) has been reported to have high chlorine resistance (55), and the other (CB3) has been observed to aggregate (54). These two works were done for very different reasons, but the findings indicated a need for further study and direct comparison of the chlorine resistance of these closely related viruses.

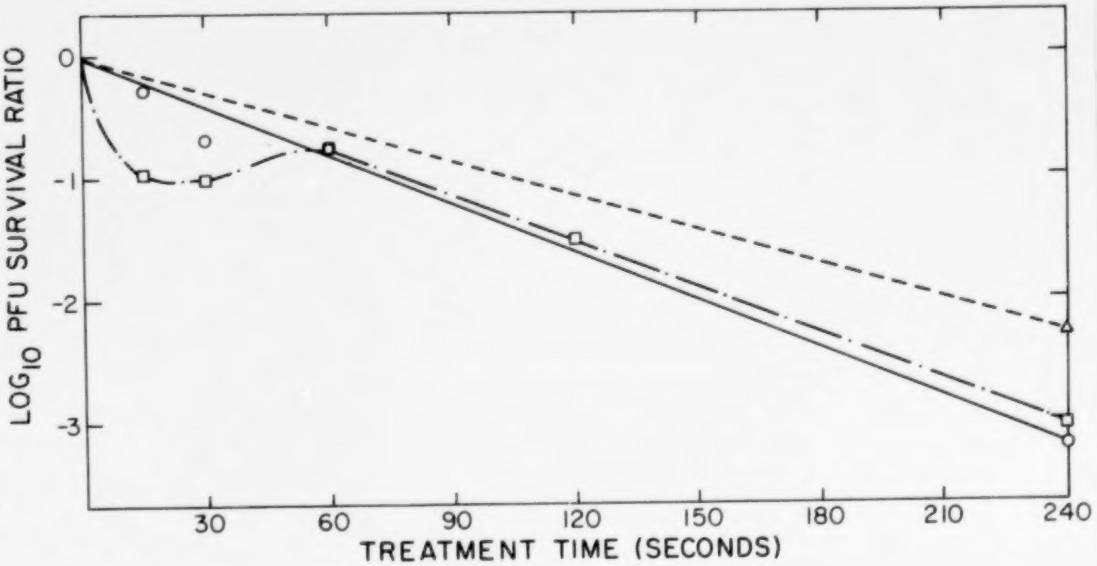


Figure 55. Inactivation of coxsackievirus B5 at 5^{o}C by 7 μM HOCl with 0.1 M NaCl present (squares), without NaCl (circles). Data of Engelbrecht et al. (55) dotted line.

Our finding that CB5 aggregates rapidly after purification, at all pH values from 3 to 10 even in the presence of 0.1 M NaCl, which has a dispersive effect on some other viruses, would seem to supply a reason for its reported high chlorine resistance were it not for the other apparently conflicting facts revealed here. The CB3 strain aggregated also in our experiments at pH 3 to 5 but not at pH 6 and above. We have seen no evidence of the aggregation of CB3 reported by Totsuka et al. (54). The chlorine resistance of the two was about the same at pH6 (compare 10^{-2}, 10^{-3}, or 10^{-4} plaque survival levels in Fig. 53 and 57). This means that the aggregated CB5 has the same chlorine resistance as the dispersed CB3 at pH6, and this is apparently true also for OCl^{-} at pH 10 (compare Fig. 54 and 58).

We were not able to measure the resistance of dispersed CB5 to chlorine and to detect the effect of aggregation directly. Only by using diethylaminoethyl dextran were we able to see this virus in dispersed from, and the minimum amount required was too much for a "demand-free" experiment. Experimental efforts to do this resulted in increased inactivation rates in spite of simultaneous loss of free chlorine from diethylaminoethyl dextran demand, possibly due to dispersion of the virus or to changes in the surface properties evident in the isoelectric focusing data. Only

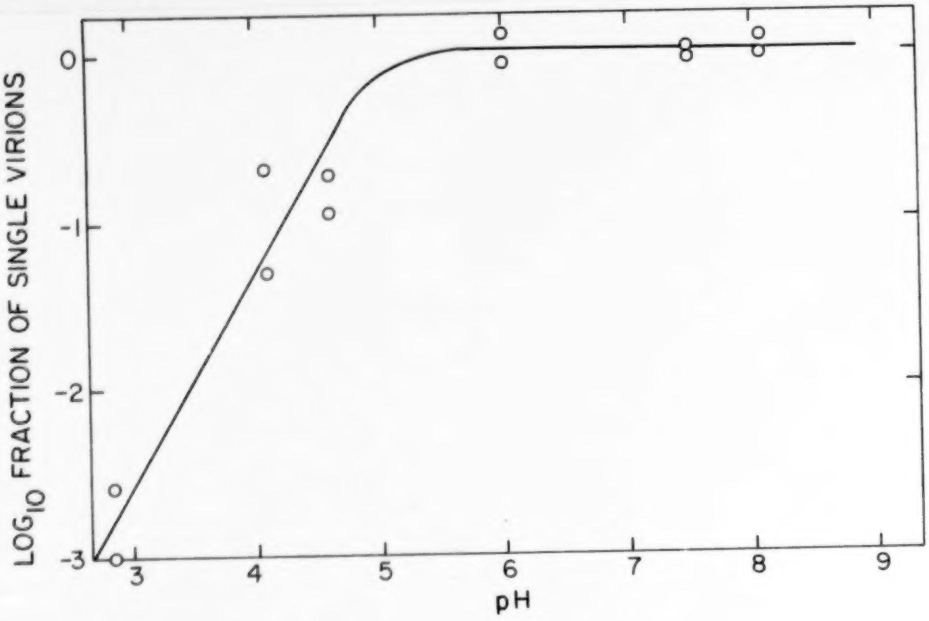


Figure 56. Aggregation of coxsackievirus B5 as a function of pH.

these meager qualitative results suggest that virion aggregation may account for part of the resistance of CB5.

Both types of the coxsackievirus differ substantially from poliovirus in their chlorine sensitivity in the presence of NaCl. At pH 6 the inactivation rate of poliovirus (Mahoney) was increased threefold when 0.1 M NaCl was added to the buffer. Neither coxsackievirus was affected in this way by NaCl at pH 6; however, both were strongly affected at pH 10. In both cases, the otherwise weakly active OCl^- ion gave rapid inactivation comparable with that of HOCl at pH6. Similar enhancing effects have been seen with NaCl and poliovirus at pH 10, and we would expect that the superiority of OCl^- over HOCl in the inactivation of poliovirus (Mahoney) reported by Scarpino et al. (18) was due to the KCl that was present in their pH 10 buffer but not in their pH 6 buffer. Our experiments with 0.1 M KCl showed its effect ot be greater than that of NaCl. Still another monovalent chloride (CSCl) was tested briefly here. It too increased the inactivation rate of CB5 at pH 10 but not to the extent observed with either the sodium or the potassium slat (Fig. 54). This suggests that the metal ion either was having some effect on the virus or it was reacting with the OCl^- and forming ion pairs which may be as effective as HOCl. No divalent ions were tested here, but some experiments with CA^{2+} by Kenyon and Schaub (51) indicate that 250 mg of $CaCl_2$ per liter increased the rate of inactivation of poliovirus in the presence of OCl^-.

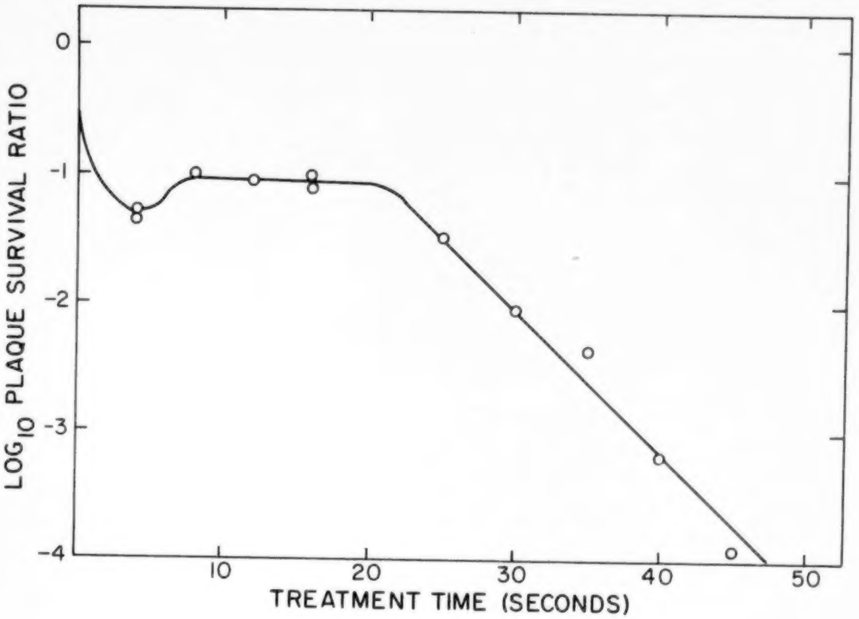


Figure 57. Inactivation of coxsackievirus B3 at 20°C by 20 µM HOCl at pH 6.

While considering Fig. 54 it is worthy of note that the virus was aggregated in all three experiments, although not necessarily to the same extent. Still, some lines are straight and others are curved. Straight lines are seen also with aggregated virus in Fig. 51 even where only 10^{-4} of the PFU survive, and curved lines have been obtained in similar experiments with well-dispersed poliovirus (22). In the presence of all these facts, the practice of deriving from the shape of a disinfection curve, the distribution of aggregate sizes in the virion suspension, becomes a doubtful activity. Apparently there are chlorine disinfection curves of a variety of shapes emerging from experiments with monodisperse virion suspensions, some of which closely resemble shapes expected from a mixture of aggregate sizes, and there are also straight lines observed in some cases in the present work where aggregation is known to exist.

The inactivation rate at pH 6 of CB5 increases linearly with increasing HOCl concentration but only up to about 25 µM, above which it remains constant (Fig. 52). Saturation effects of this kind have been observed with several picornaviruses and several halogen agents as we have already seen.

Transient departures from linear semilog disinfection rate plots such as those of Fig. 53 (CB5, pH 6, 0.1 M NaCl present) and Fig. 57 (CB3, pH 6, no salt added) are not yet understood. They have been seen before with echovirus and with poliovirus (24,51). Further study of this phenomenon is

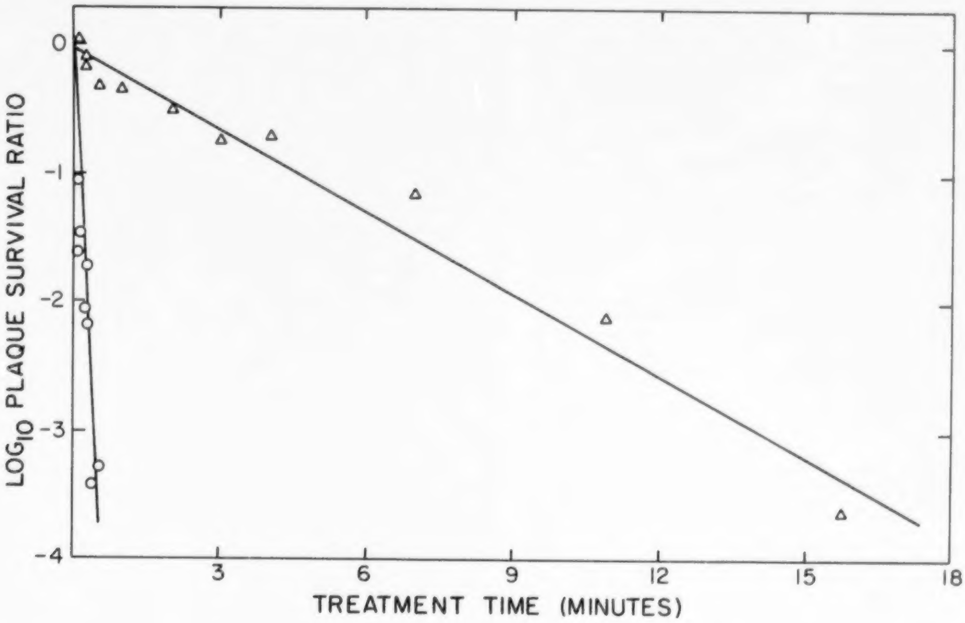


Figure 58. Inactivation of coxsackievirus B3 at 20°C by 20 μM OCl^- at pH10 (triangles) and the rapid reaction when 0.1 M NaCl was added to the buffer (circles).

continuing in this laboratory in the hope that it will reveal something of the mechanism of the disinfection process. At present, it seems to be of little practical significance because the time involved is usually small, but the frequency of its appearance in recent chlorine work suggeste that the simple straight line may be the exception rather than the general rule among viruses.

Doubtless the item of greatest practical significance in this work on coxsackievirus is the observation previously reported for poliovirus (44) that the comparatively weak disinfecting action of OCl^- at pH 10 is enhanced many times (in this case20-to 30-fold) by the addition of 0.1 M NaCl. Under these conditions these viruses are destroyed at a rate comparable with that of the same amount of free chlorine, as HOCl, at pH 6. The effectiveness of the three different chlorides that have been tested in this respect are similar but different in magnitude, with CsCl the least, NaCl the next, and KCl the most active. The large heavily hydrated cesium ion might be expected to have the greatest difficulty penetrating the virus capsid, but the reason for the difference between the effects of Na^+ and K^+ must be sought elsewhere. A difference in ion pairing has been demonstrated here, with the potassium ion showing a greater tendency to pair with OCl^- than sodium. If the pairs are more active in the inactivation of virus than the ions, as suggested by Sugam and Helz (49), the explanation may be at hand.

TABLE 6. Calculated extent of ion pair formation between OCl^- and Na^+ or K^+

Salt conc (m)	pH	Amperometric Cl Clτ (μM)	Absorbance	ε	% OCl involved in ion pairing
NaCl					
0.50	9.98	185	0.0505	323	7.7
0.10	9.95	175	0.0585	333	4.9
0.05	9.94	157	0.0540	345	1.4
KCl					
0.50	9.92	179	0.0560	314	10.3
0.10	9.95	191	0.0620	326	6.9
0.05	9.93	186	0.0615	332	5.1

SERIES IX. INACTIVATION OF SOME OF THE SAME VIRUSES BY ULTRAVIOLET RAYS

Some of the results obtained in chlorine inactivation experiments with both of these viruses have raised questions regarding possible changes in the sensitivity of the virus during the inactivation process, when the pH is changed and when salt (NACl) is added to the buffer. It seemed worthwhile to learn whether such changes occurred when an entirely different inactivating agent was employed, one whose action was primarily on the RNA of the virion.

A low pressure mercury discharge lamp was used (Westinghouse "Odorout") from which the radiant flux at 2537Au was adjusted to $52 \mu W/cm^2$, at the surface of a quartz-bottomed treatment chamber holding the virus suspension in a layer 2 mm deep. The virus suspension was diluted beyond the point where any absorption could be detected with the radiometer as the rays passed through the thin liquid layer, so the incident UV intensity did not vary appreciably as it passed through; all virions received essentially the same intensity of rays. Under these conditions at room temperature (25°C) the rate of inactivation of both viruses was slow enough to permit easy sample taking for plaque titration of survivors.

The results with poliovirus (Mahoney) are shown for virus suspended in dilute phosphate-carbonate buffer at pH 6 (Fig. 59) at pH 7 (Fig. 60), at pH 8 (Fig. 61), at pH 9 (Fig. 62) and at pH 10 (Fig. 63). Apparently the photoinactivation of this virus is little if any dependent on pH, for no substantial systematic changes occurred as the pH was raised successively in a series of experiments from 6 to 10. Nor did the suspension of the virus in physiological saline (0.14M NaCl) at pH 7 make any substantial change in the inactivation rate (Fig. 64).

Ultraviolet inactivation experiments with the echovirus showed none of the transient variations in rate that were observed in the HOCl experiments. They were done exactly like those with the poliovirus. At pH 6 with the virus suspended in 0.14 M NaCl the rate (Fig. 65) was slightly slower than that of the poliovirus and perhaps slightly curved but otherwise quite uneventful. The rate was not changed by inducing aggregation by acid treatment (Fig. 66).

These seven experiments indicate that the photosensitive target of the poliovirus, presumably the genome, is not altered by change in pH in the range 6 to 10, nor is it changed by addition of NaCl at physiological concentration. Neither is there any transient change in inactivation rate with ultraviolet rays as there was when echovirus was exposed to 20 μM HOCl at pH 6. While it cannot be said with certainty that photoinactivation of these viruses is entirely an RNA reaction nor that the HOCl acts entirely on the protein coat, the suggestion is strong that the anomalies observed

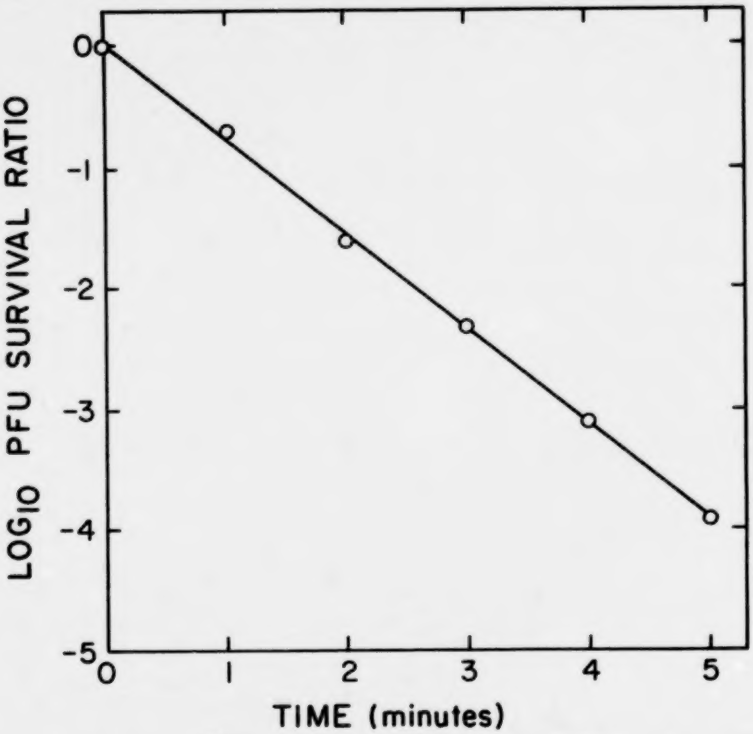


Figure 59. Inactivation of poliovirus (Mahoney) at pH6 by ultraviolet rays.

with chlorine are associated with the capsid protein.

A "CHLORINE-RESISTANT" STRAIN OF POLIOVIRUS

Bates et al. (56) have exposed their preparation of poliovirus to chlorine in a series of ten experiments. Survivors of each exposure were increased by growth in cells then exposed again. We have examined this end-product and the electron micrographs were difficult to interpret. There was so much foreign matter present that virus clumps, if they are present, could not be recognized with any degree of certainty.

Exposure of this virus preparation to HOCl at pH 7 in the continuous flow apparatus under conditions exactly like those we have used for our purified Mahoney virus has shown an inactivation rate decreasing with time in a manner characteristic of aggregated preparations in general. So it appears that some of the larger particles that were seen in the pictures were, indeed, virion aggregates. This conclusion was strengthened when, using some of the starting material sent to us, we inoculated HEp2 cell cultures and purified the progeny virus in the same way that our virus has been purified. With this preparation there was no continuous reduction in

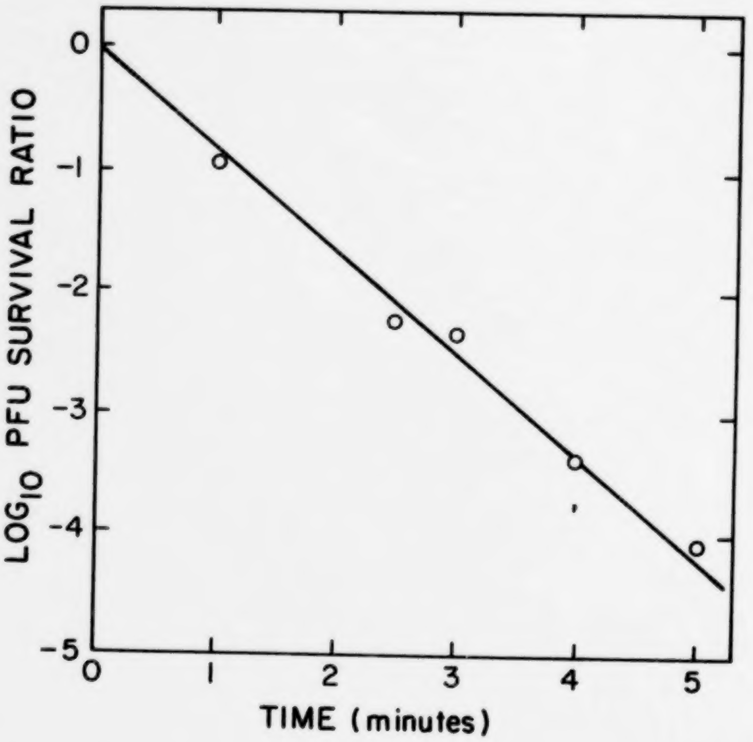


Figure 60. Inactivation of poliovirus (Mahoney) at pH7 by ultraviolet rays.

activation rate with HOCl. There was, however, a slower rate of inactivation than we have previously reported for our Mahoney type 1 virus, slower by a factor of two. We do not regard a factor of 2x as a very significant rate difference and we do not regard our few EM pictures as sufficient evidence for lack of an aggregation effect as claimed by Schaffer et al who used our picture in a subsequent publication (57). While it is true that no large aggregates were seen in the EM pictures it must be remembered that only a very minute sample was examined and no careful experiments were done, as they were earlier with reovirus, to learn whether surving PFU sedimented in the centrifuge as single virions or as aggregates. We suspect that they were aggregates. We are not convinced that the selective pressure applied has brought out a chlorine-resistant poliovirus.

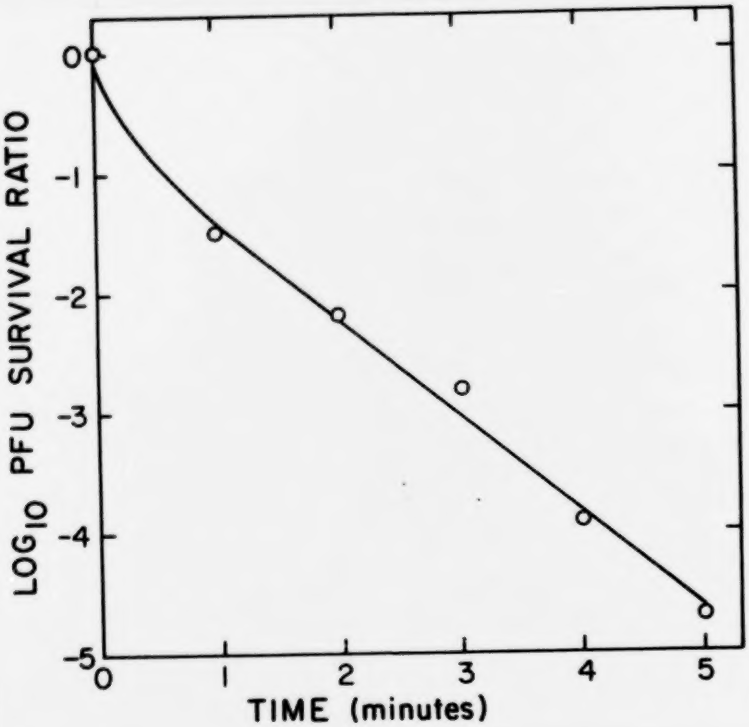


Figure 61. Inactivation of poliovirus (Mahoney) at pH 8 by ultraviolet rays.

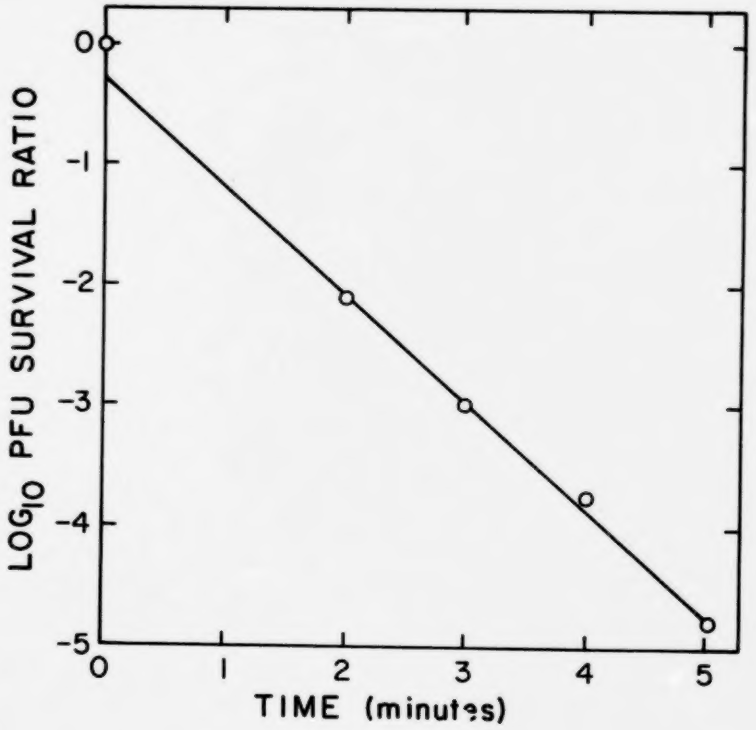


Figure 62. Inactivation of poliovirus (Mahoney) at pH 9 by ultraviolet rays.

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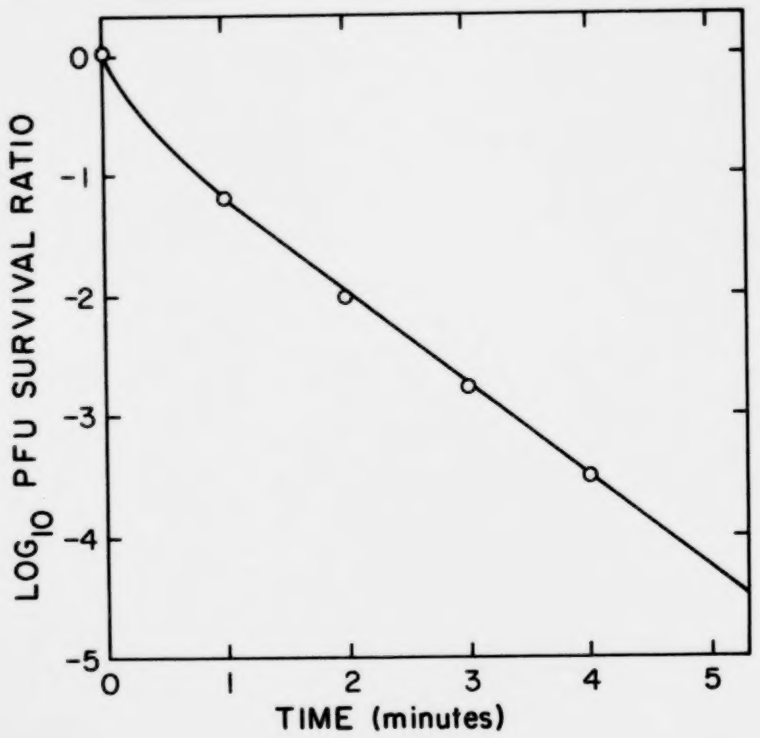

Figure 63. Inactivation of poliovirus (Mahoney) at pH 10 by ultraviolet rays.

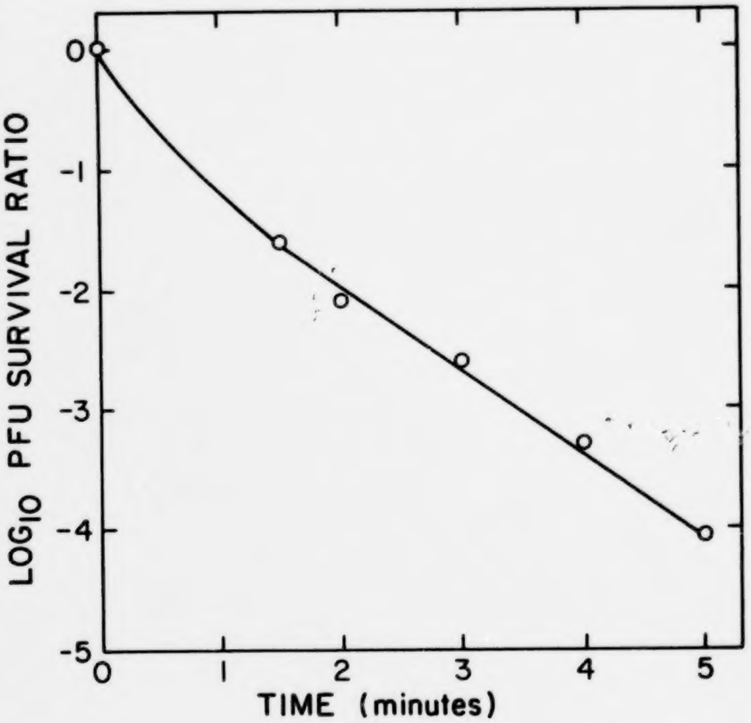

Figure 64. Inactivation of poliovirus (Mahoney) in 0.14 M NaCl by ultraviolet rays.

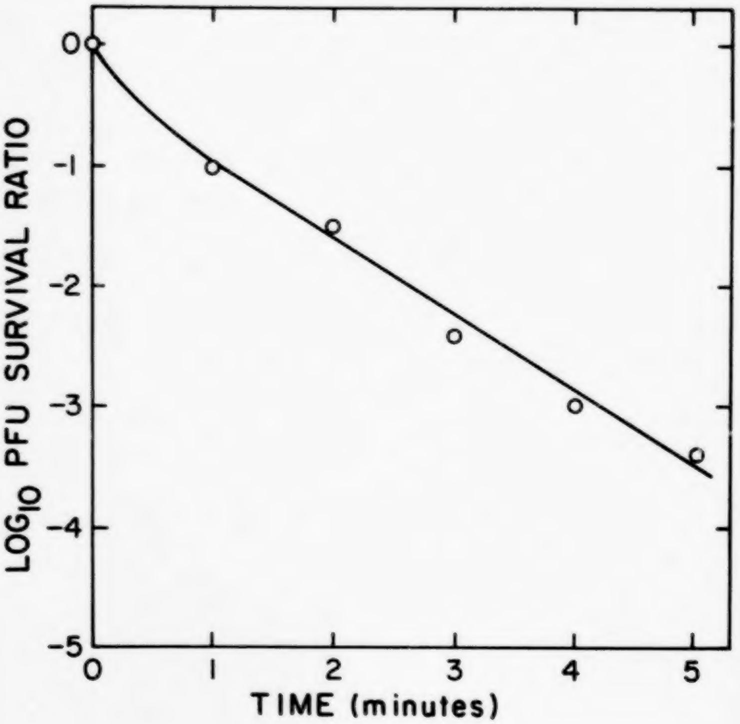

Figure 65. Inactivation of echovirus (Farouk) in 0.14 M NaCl by ultraviolet rays.

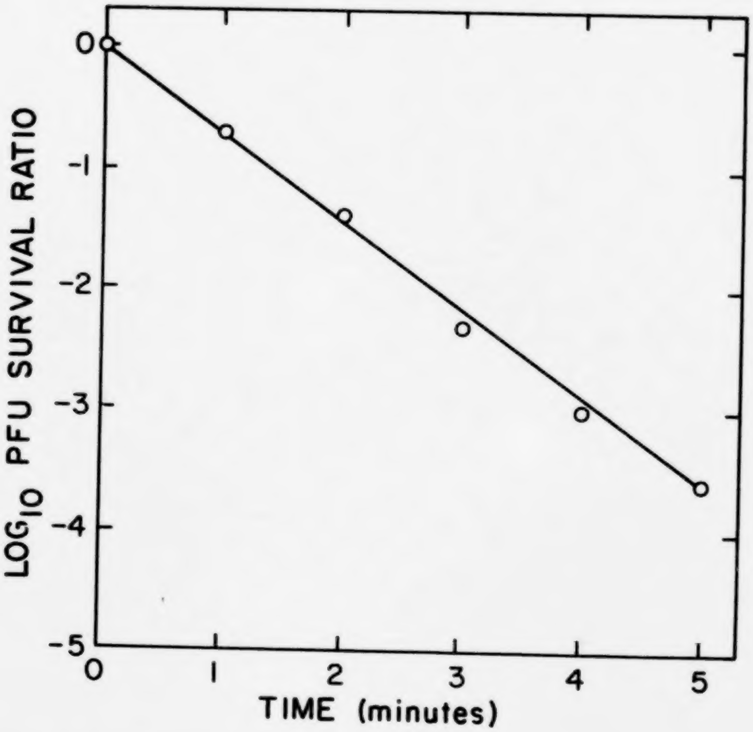

Figure 66. Inactivation of echovirus (Farouk) at pH6 by ultraviolet rays.

REFERENCES

1. Sharp, D.G. 1976. Virus Particle Aggregation and Halogen Disinfection of Water Supplies. Environmental Protection Tech. Series 600/2-76-287.

2. Sharp, D.G., R. Floyd and J.D. Johnson. 1975. Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine. Appl. M'biol. 29:94-101.

3. Sharp, D.G., R. Floyd and J.D. Johnson. 1976. Initial Fast Reaction of Bromine on Reovirus in Turbulent Flowing Water. Appl. and Env. M'biol. 31:173-181.

4. Floyd, Roger, J.D. Johnson and D.G. Sharp. 1976. Inactivation by Bromine of Single Poliovirus Particles in Water. Appl. and Env. M'biol. 31:298-303.

5. Young, Dorothy C., and D.G. Sharp. 1977. Poliovirus Aggregates and their Survival in Water. Appl. and Env. M'biol. 33:168-177.

6. Sharp, D.G. 1974. Physical Assay of Purified Viruses, Particularly the Small Ones. C.J. Arceneaux (ed.) Proceedings of the 32nd Annual Meeting of the Electron Microscopy Society of America. Claitor's Publishing Div., Baton Rouge, La. pp. 264-265.

7. Floyd, Roger and D.G. Sharp. 1977. Aggregation of Poliovirus and Reovirus by Dilution in Water. Appl. and Env. M'biol. 33:159-167.

8. Floyd, Roger and D.G. Sharp. 1978. Viral Aggregation: Quantitation and Kinetics of the Aggregation of Poliovirus and Reovirus. Appl. and Env. M'biol. 35:1079-1083.

9. Kelley, C.M. and H.V. Tartar. 1956. On the System: Bromine-Water. J. Am. Chem. Soc. 78:5752-6.

10. Griffith, R.O., A. McKeown and A.G. Winn. 1932. The Bromine-Tribromide Equilibrium. Trans. Faraday Soc. 28:101-107.

11. Kolthoff, I.M. and J.J. Lingane. 1952. Polarography, Vol. II 2nd ed., Interscience, New York, N.Y., p. 946.

12. Johnson, J.D. and R.J. Overby, 1969. Stabilized Neutral Orthotholidine, SNORT, Colorimetric Method for Chlorine. Anal. Chem. 41:1744-50.

13. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Association, Washington, D.C. 1975.

14. Floyd, Roger, J.D. Johnson and D.G. Sharp. 1978. Inactivation of Single Poliovirus Particles in Water by Hypobromite ion, Molecular Bromine, Dibromamine and Tribromamine. Env. Sci. and Tech. 12:1031-1035.

15. Sharp, D.G. and M.J. Buckingham. 1956. Electron Microscopic Measure of Virus Particle Dispersion in Suspension. Biochem. et Biophys. Acta 19:13-21.

16. Weidenkopf, S.J. 1958. Inactivation of Type I Poliomyelitis Virus with Chlorine. Virology. 5:56-67.

17. Morris, J.C. 1975. In Disinfection-Water and Wastewater, J.D. Johnson, ed., Ann Arbor Science, Ann Arbor. Mich. pp. 1-10.

18. Scarpino, P.V., G. Berg, S.L. Chang, D. Dahling and M.Lucas. 1972. A Comparative Study of the Inactivation of Viruses in Water by Chlorine. Water Research 6:959-965.

19. Symons, J.M. and J.C. Hoff. 1975. Rational for Turbidity Maximum Contaminant Level. Proc. AWWA Water Quality Tech. Conf. AWWA Research Foundation Publishers. Denver, Col., Paper #2A-4a.

20. Berg., G., S.L. Chang and E.K. Harris. 1964. Devitalization of Microorganism by Iodine 1. Dynamics of the Devitalization of Enteroviruses by Elemental Iodine Virology 22:469-481.

21. Taylor, D.G. and J.D. Johnson, 1974 in Chemistry of Water Supply, Treatment and Distribution. A.J. Rubin, Ed., pp. 369-408. Ann Arbor Science Publishers, Ann Arbor, Mich.

22. Floyd, Roger, D.G. Sharp and J.D. Johnson. 1979. Inactivation by Chlorine of Single Poliovirus Particles in Water. Env. Sci and Tech. 13:438-442.

23. Kott, Y., E.M. Nupen and W.R. Ross. 1975. Effect of pH on the Efficiency of Chlorine Disinfection and Virus Enumeration. Water Res. 9:869-872.

24. Young, Dorothy C., J.D. Johnson and D.G. Sharp. 1977, The Complex Reaction Kinetics of ECHO-1 Virus with Chlorine in Water. Proc. Soc. Exp. Biol. and Med. 156:496-499.

25. von Smoluchowski, M. 1917. Versuch einer Mathematischen Theorie der Koagulationskinetic Kolloider. Z. Phys. Chem. 92:129-168.

26. Mandel, B. 1971. Characterization of Type I poliovirus by electrophoretic analysis. Virology 44:554-568.

27. Fujioka, R.S. and W.W. Ackermann. 1975. Evidence for Conformational States of Poliovirus: Effects of Cations on Reactivity of Poliovirus to Guanidine. Proc. Soc. Exp. Biol. & Med. 148:1070-1074.

28. Young, Dorothy C. and D.G. Sharp. 1979. Partial Reactivation of Chlorine-Treated Echovirus. Appl. and Env. M'biol. 37:766-773.

29. Berg, G., R.M. Clark, D. Berman and S.L. Chang. 1967. Aberations in Survival Curves, pp. 235-240 in G. Berg (ed.). Transmission of Viruses by the Water Route. Interscience Publishers Inc., New York.

30. Ward, R.L. 1978. Mechanisms of Poliovirus Inactivation by Ammonia. Appl. Env., M'biol. 26:299-305.

31. Abel, P. 1962. Multiplicity Reactivation and Marker Rescue with Vaccinia Virus. Viology 17:511-519

32. Dalton, D., K.S. Kim and D.G. Sharp. 1967. Multiplicity Activation of Vaccinia Virus in L cells. Proc. Natl. Acad. Sci. USA 58:1758-1761.

33. Setlow, J.K. 1964. Effects of UV on DNA: Correlations among Biological Changes and Repair Mechanisms. Photochem. Photobiol. 3:405-410.

34. Rupert, C.S. and W. Harm. 1966. Reactivation after Photobiological Damage. Adv. Rad. Biol. 2:1-63.

35. Fujioka, R.S. and W.W. Ackermann. 1975. The Inhibitory Effects of $MgCl_2$ on the Inactivation Kinetics of Polivirus by Urea. Proc. Soc. Exp. Biol. and Med. 148:1063-1069.

36. Sharp, D.G. and Ruth C. Dunlap, 1966. Multiplicity Reactivation Vaccinia Virus in the Cells of the Chorioallantoic Membrane. Proc. Soc. Exp. Biol. and Med. 123:111-114.

37. Hartman, R.J. 1947. Colloidal Chemistry. Haughton Mifflin Co., Cambridge, pg. 270.

38. Floyd, Roger and D.G. Sharp. 1978. Viral Aggregation: Effects of Salts on the Aggregation of Poliovirus and Reovirus at Low pH. Appl. and Env. M'biol. 35:1084-1094.

39. Stern, O. 1924. zur Theorie der Elektrolytischen Doppelschicht. Zeit, Elektrochim. 30:508-516.

40. Thomas, A.W. 1934. Colliod Chemistry. McGraw-Hill Co., New York.

41. Chlumecka, V., P.D. Obrenan and J.S. Colter. 1973. Electrophoretic Studies on Three Variants of Mengo Encephalomyelitis virus. Can. J. Biochem. 51:1521-1526.

42. Floyd, Roger and D.G. Sharp. 1979. viral Aggregation: Buffer Effects in the Aggregation of Poliovirus and Reovirus at Low and High pH. Appl. and Env. M'biol. 38:395-401.

43. McIlvaine, T.C. 1921. A Buffer Solution for Colorimetric Comparison. J. Biol. Chem. 49:183-196.

44. Sharp, D.G., Dorothy C. Young, Roger Floyd and J.D. Johnson 1980. Effect of Ionic Enviromnment on the Inactivation of Poliovirus in Water by Chlorine. Appl. and Env. M'biol. 39:530-534.

45. Boeye, A. and A. van Elsen. 1967. Alkaline Disruption of Poliovirus: Kinetics and Purification of RNA-Free Particles. Virology 33:335-343.

46. Salo, R.J. and D.O. Cliver. 1976. Effect of pH, Salts and Temperature on the Infectivity and Physical Integrity of Enteroviruses. Arch. Virol. 52:269-282.

47. Davies, C.W. 1962. Ion Association. Butterworth & Co. London, pg. 39.

48. Morris, J.C. 1966. The Acid Ionization of HOCl from 5° to 35°C J. Phys. Chem. 70:3798-3805.

49. Sugam, R.E. and G.R. Helz. 1976. Apparent Ionization Constant of HOCl in Sea Water. Env. Sci. Tech. 10:384-386.

50. Sharp, D.G. and Jenny Leong. 1980. Inactivation of Poliovirus I. (Brunhilde) Single Particles by Chlorine in Water. Appl. and Env. M'biol. 40:381-385.

51. Kenyon, K.F. and S.A. Schaub. 1978. Free Available Chlorine Disinfection of Monodisperse Enteric Microorganisms. Am. Chem. Soc. Div. Env. Chem. Prepr. 18:201-204.

52. Sharp, D.G. 1967. Electron Microscopy and Virus Particle Function. In G. Berg (ed.) Transmission of Viruses by the Water Route, pp. 193-217. Interscience Publishers, New York.

53. Jensen, H., K. Thomas and D.G. Sharp. 1980. Inactivation of Coxsackieviruses B3 and B5 in Water by Chlorine. Appl. and Env. M'biol. 40:633-640.

54. Totsuka, A., K. Ohtaki and I. Tagaya. 1978. Aggregation of Enterovirus Small Plaque Variants and Polioviruses Under Low Ionic Strength Conditions. J. Gen. Virol. 38:519-533.

55. Engelbrecht, R.S., M.J. Weber, C.A. Schmidt and B.L. Salter, 1978. Virus Sensitivity to Chlorine Disinfection of Water Supplies. U.S. Env. Protection Agency Report 600/2-78-123.

56. Bates, R.C., P.T.B. Shaffer and S.M. Sutherlan. 1977. Development of Poliovirus Having Increased Resistance to Chlorine Inactivation. Appl. and Env. M'biol. 34:849-853.

57. Shaffer, Peter T., Theodore G. Metcalf and Otis J. Sproul. 1980. Chlorine Resistance of Poliovirus Isolates Recovered from Drinking Water. Appl. and Env. M'biol. 40:1115-1121.